# THE USE OF EPIDEMIOLOGY FOR EVALUATING THE PERFORMANCE AND ADMINISTRATION STRATEGIES OF TOPICAL DRUGS FOR CONTROLLING SEA LICE IN CHILE

A Thesis
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University of Prince Edward Island

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Charlottetown, P.E.I
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*To Natalia, Eduardo and Javiera, my reasons to live*

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## ABSTRACT

Sea lice is considered the most important ectoparasite that affects salt water farmed salmonids around the world. Its high economic impact has motivated efforts for its control. *Caligus rogercresseyi* is the sea lice species of major economic importance for the salmon industry in Chile. Sea lice control in Chile is largely based on chemotherapeutants administered as immersion treatments, synthetic pyrethroids and organophosphates being the most used drugs at the present. In recent years, farmers have reported decreased performance of pyrethroid-based treatments. Main causes for treatment failures may be low sensitivity of sea lice to the anti-parasitic drug, failures in the administration of the product, or re-infestation from external sources (i.e. neighbouring farms) after the treatment, especially in areas with intensive salmon farming. The general objective of this thesis was to evaluate the performance of topical drugs administered as immersion treatments and to evaluate area-level treatment strategies using field data under an epidemiological approach which considered the multi-factorial nature of the sea lice dynamics at the farm and the farm management area level. The specific objectives of this thesis were: 1) to assess the performance of the synthetic pyrethroids deltamethrin and cypermethrin on different life stages of *C. rogercresseyi* (Chapter 2), 2) to explore the spatial and spatio-temporal variation of *C. rogercresseyi*'s response to pyrethroid treatments and examine factors related to this variability (Chapter 3), and 3) to evaluate the effect of treatment synchronization on adult sea lice levels up to 8 weeks after treatment (Chapter 4).

In Chapters 2 and 3 we used the sea lice level one week after treatment as the variable of interest, while in Chapter 4 we included sea lice levels from week 2 to 8 after the procedure. Pre-treatment sea lice levels, environmental and management factors were included as predictors in the three cases. In Chapter 2 we compared the treatment performance between three pyrethroid based products available for the Chilean industry and with a negative control (i.e. non-treated pen). In Chapter 3 we calculated residuals at the treatment level and farm effects from a multi-level regression model. Farm effects were subjected to a

global cluster analysis (Moran's *I*) and to a purely spatial analysis in order to detect clusters of high sea lice levels in the study area. Residuals at the treatment level were included in a spatio-temporal analysis. In Chapter 4 we tested the effect of three different measures of treatment synchronization on the adult lice levels up to 8 weeks after treatment.

Pens treated with any of the three pyrethroid products had significantly lower mean juvenile, mobile adult, and gravid female sea lice abundance after treatment compared to the untreated pens; however, the effect on juvenile lice was less than on mobile stages. No significant differences were observed in the numbers of juvenile, adult male, and non-gravid female when the three products were compared to each other; however, cypermethrin exhibited a small, yet significantly greater effect on the gravid female group.

The global cluster analysis revealed treatment performance was clustered in space, while the scan spatial statistic found two areas where the post-treatment adult lice level attributed to the farm effect was significantly higher than in the rest of the study area. These spatial clusters remained even once we adjusted for environmental and management predictors. In the spatio-temporal analysis, we found three clusters; however, they become non-significant when predictors were included in the model.

The model containing the treatment synchronization variable, expressed as the number of farms within a window of 1 week and 10 km seaway distance that reported treatment against sea lice, fitted the model the best. Higher intensity of treatment synchronization was significantly associated with lower adult lice levels at weeks 5 and 6 after treatment. This relationship appeared to be linear, suggesting that higher levels of synchronization may result in lower sea lice levels at these weeks.

Findings of this thesis suggest that during 2011 and 2012 approved pyrethroids in Chile were more effective on adult than juvenile lice. In general, the three products in evaluation showed a similar performance, but cypermethrin was associated with better results in the gravid female lice group.

We also found treatment performance was clustered in space, and two areas of poor treatment

performance were located. These spatial clusters remained even once we adjusted for environmental and management predictors, suggesting unknown factors were causing the clustering in these areas. Potential factors that may explain these clusters include lower sensitivity of sea lice populations to pyrethroids and problems with the application of the product; however, further research is needed to clarify this situation. With respect to strategies of treatment at the area level, our results suggest that treatment synchronization reduced adult lice levels between weeks 5 and 6 after treatment, which could mean a two-week delay in the rise of sea lice levels after a treatment.

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Finally, to my beloved and courageous Natalia for have laughed and cried with me during this time. This achievement is as much yours as mine.

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## List of Abbreviations

| | |
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| AChE | Acetylcholinesterase |
| AIC | Akaike Information Criteria |
| AR(1) | First order autoregressive correlation structure for residuals |
| BLUPs | Best linear unbiased predictors |
| CM | Cypermethrin |
| DM | Deltamethrin |
| EB | Emamectin benzoate |
| GSTs | Glutathione-S-transferases |
| ICC | Intra-class correlation coefficient |
| Intesal | Instituto Tecnológico del Salmón |
| IPM | Integrated pest management |
| LRT | Likelihood ratio test |
| MFOs | Mixed function oxidases |
| MHC | Major histocompatibility complex |
| ML | Maximum likelihood |
| MSP | Maximum synchronization potential |
| NB | New Brunswick, Canada |
| NRP | Reproduction potential of the neighbouring farms |
| REML | Restricted maximum likelihood |
| SCTs | Strategic coordinated treatments |
| SEARCH | Sea Lice Resistance to Chemotherapeutants Project |
| TSI_1 | Treatment synchronization intensity |
| TSI_2 | Alternative treatment synchronization variable 2 |
| TSI_3 | Alternative treatment synchronization variable 3 |

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# CHAPTER 1
# GENERAL INTRODUCTION

## 1.1. Background

The Chilean salmon industry represents an important economic sector at the provincial and national levels. In 2013, this industry produced 786,091 tons of farmed salmonids of which 63% was Atlantic salmon, 19% Coho salmon and 18% rainbow trout (Sernapesca, 2014). Production of Chinook salmon was marginal. Projections for 2014 indicate the total production will surpass 800 thousand tons. In 2013, this industry accounted for 3.5% of total exports of the country (DIRECON, 2014), and consisted of about 500 companies that employ more than 50,000 people, including salmon growing, salmon processing, and service companies (CONICYT, 2012). During 2013, more than 490 fish farms (fresh or sea water facilities) reported commercial activity (Sernapesca, 2014). Internationally, Chile accounts for about 30% of world production of farmed salmon (Sernapesca, 2014; FAO, 2014). Salmonid species of major commercial importance in Chile are Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and Coho salmon (*Oncorhynchus kisutch*).

As in any other salmon farming industry around the world (Jones et al, 2014), infectious diseases have been major constraints to the sustainable development of the Chilean salmon industry (OECD, 2009; Alvial et al., 2012). In particular, sea lice have been considered one of the most challenging fish health problems for the following reasons: 1) complex control, as the sea lice stages have different sensitivities to chemotherapeutants, and, more recently, cases of low sensitivity and resistance of sea lice populations to particular agents have been reported, consequently, performance of delousing drugs is not always as expected; and 2) in areas where fish farming activity is intense, sea lice is ubiquitous in sea water, therefore, despite the control measures taken at the farm, new sea lice will parasitize fish.

## 1.2. Sea lice

### 1.2.1. General biology

Sea lice is the common name given to a group of copepod ectoparasites parasites, from the family

Caligidae, which affect fish in salt water (Burka et al., 2012; Torrissen et al., 2013). This family of parasites accounts for more than 60% of reports of parasites in fish in marine environments (Johnson et al., 2004). Among Caligids, *Lepeophtheirus salmonis* and *Caligus rogercresseyi* are the main species affecting both farmed and wild salmonids (Burka et al., 2012).

*L. salmonis*, which is found only in the northern hemisphere, affects predominantly salmonids in the genera *Salmo*, *Oncorhynchus*, and *Salvelinus*, including farmed Atlantic salmon (Pike & Wadsworth, 1999). *Oncorhynchus kisutch* (Coho salmon) and *Oncorhynchus gorbuscha* (pink salmon) have been described to be more resistant to *L. salmonis* infections than other salmonids species (Fast et al., 2003; Johnson et al., 2004; Jones et al., 2007; Wagner et al., 2008). Among the susceptible species, Atlantic salmon (*Salmo salar*) is the more affected salmonid by *C. rogercresseyi* (Bravo, 2003). *Caligus elongatus* can also be found on salmonids in the Northern Hemisphere, but it has been less of a problem for farmed fish than *L. salmonis* (Costello, 2006). *L. salmonis* has been the responsible agent for important disease outbreaks in Canada, Faroe Islands, Ireland, Maine (USA), Norway, and Scotland (Costello, 2006; Burka et al., 2012; Torrissen et al., 2013).

*C. rogercresseyi* is the most important species of sea lice in Chile for the salmon industry. In contrast to *L. salmonis*, *C. rogercresseyi* has a lower host specificity, as it has been found in local wild species, such as the native rock cod (*Eleginops maclovinus*) and the sea silverside smelt (*Odontesthes regia*), as well as the introduced Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Carvajal et al., 1998). Coho salmon (*Oncophynchus kisutch*) has been reported to be less susceptible than other salmonid species (Bravo, 2003).

*Caligus rogercresseyi* was described in 2000 by Boxshall and Bravo, though it was originally reported as *Caligus flexispina* (Carvajal et al., 1998; Boxshall and Bravo, 2000). Because the first introduced salmonids in Chile were free of sea lice, it is believed *C. rogercresseyi* was transmitted from native

species naturally infected by this parasite and commonly found near salmon farms (Carvajal et al., 1998). *C. rogercresseyi* can be found along the southern Pacific coast of Chile (41° S), except for the far south (51° S), where low water temperatures prevent the sea lice development (S. Bravo, pers. comm.).

### 1.2.2. Life cycle

The life cycle of sea lice comprises both free swimming planktonic and parasitic phases. The planktonic phase consists of two nauplii stages and one copepodid stage, the latter being the first infective stage. The planktonic stages of *L. salmonis* have around 7 days to find a host, although this time is temperature and species dependent (Stucchi et al., 2011). The copepodid finds its host by following behavioural traits, including gradients of host-derived chemicals (e.g. kairomones), and vibrations of host origin (Heuch et al., 1995; Heuch & Karlsen, 1997; Bailey et al., 2006). The copepodid attaches to the fish skin using its hooked pair of antennae; this process is mediated by chemo-receptors in copepodid's antennules (Gresty et al., 1993). During its developmental process, the parasite gradually increases in size and is attached at all times to the fish. Once on the fish, the copepodid moults into chalimus I, and develops a frontal filament to attach itself to the fish (Johnson & Albright, 1991; González & Carvajal, 2003). The *C. rogercresseyi* parasite moults into three more chalimus stages (II, III and IV) while attached to the fish, while in the case of *L. salmonis* (González & Carvajal, 2003), recent research have reported only two chalimus stages (I and II) before the louse develops to the pre-adult stage (Hamre et al., 2013). Finally, the individual matures into an adult stage (González & Carvajal, 2003). Sexual dimorphism can be seen at the chalimus III stage; however, is clearer at chalimus IV. In the case of *L. salmonis*, two pre-adult stages have been described after the chalimus stages and before the adult stage (Johnson & Albright, 1991; Boxhall & Bravo, 2000; González & Carvajal, 2003). Mean sizes for developmental stages of *C. rogercresseyi* are: nauplii 0.45 mm long, copepodid 0.66 mm long, chalimii 1.85 mm long, and adult 4.81 mm long (González & Carvajal, 2003).

The reproductive potential of sea lice is significant. For both sea lice species, one female louse can produce up to 11 generations of egg strings after a single copula which can take 74 days in the case of *C. rogercresseyi* (Heuch et al., 2000; Bravo, 2010). Each egg string can hatch around 30 eggs in the case of *C. rogercresseyi*, while in *L. salmonis* this number can reach 285 (Heuch et al., 2000; Bravo, 2010). Multiple copulation and polyandry have been described for *L. salmonis* (Todd et al., 2005; Costello, 2006), but not for *C. rogercresseyi*. Female *C. rogercresseyi* have demonstrated survival up to 79 days (Bravo, 2010), while female *L. salmonis* reach 191 days (Heuch et al., 2000).

González & Carvajal (2003) determined that the duration of the life cycle of *C. rogercresseyi* is dependent on the water temperature, and found that at 16.7° C the cycle is completed in 18 days, and in 45 days at 10.3° C. In the same study, it was determined that the minimum temperature at which the parasite can develop is 4.2° C. Later, Bravo (2010) estimated the duration of each stage in degree-days (see Table 1.1) and found that the first egg string is produced at 389 degree days (°D) after egg incubation, and the periodicity at which egg strings are produced depends on water temperature. This latter feature has also been described for *L. salmonis* (Heuch et al., 2000).

Sea lice are organisms whose optimal development occurs in high-salinity waters. In particular, it has been described that larval stages are the most sensitive to low salinity; that larvae are impacted in their attachment success rates. There is evidence that salinity values below 20‰ seriously impair both *L. salmonis* and *C. rogercresseyi* development (Pike & Wadsworth, 1999; Brooks et al., 2005; Genna et al., 2005; Costello, 2006; Bravo et al., 2008a). However, there is also evidence that in some cases sea lice have adapted to low-salinity waters (Bravo et al., 2008a). In addition, it has been described that *C. rogercresseyi* females tolerate lower salinity levels than males (Bravo et al., 2008a).

### 1.2.3. Lice-fish interaction

Sea lice infections produce a generalized chronic stress response. The areas of the fish where sea lice are

commonly found are those with less hydrodynamic disturbance, such as the base of fins (Genna et al., 2005). Damage is caused by the attachment to the host and feeding on the epidermal mucus, skin, and blood (González et al., 2000; Johnson et al., 2004). Once settled on the host, the parasite secretes enzymes for digestion of mucus, which helps the louse to feed and suppress the salmon's immune response at the attachment site (Ross et al., 2000). Pathological changes in the skin include loss of epithelium, bleeding, increase in mucus secretion, change in the chemical composition of mucus, tissue necrosis, and the consequent loss of function of the skin as a physical and microbiological barrier (Costello, 2006).

Infected fish have reduced appetite, growth, and food-conversion efficiency, while the stress and wounds caused by the parasite expose the fish to secondary infections due to impaired immunocompetence (Costello, 2006). It has been reported that infestation with high levels of sea lice may cause death of the fish, as a consequence of osmoregulatory stress generated by skin lesions of the host (Johnson et al., 2004; Costello, 2006). However, the precise number of lice required for these effects were not identified by the authors. Furthermore, sea lice are a potential vector for transmission of bacterial and viral diseases (Costello, 2006; Valdes-Donoso et al., 2013; Oelckers et al., 2014).

Observational and experimental evidence have shown that rainbow trout are more susceptible to *C. rogercresseyi* than Atlantic salmon, while Coho salmon is the more tolerant species (González et al., 2000; Zagmutt-Vergara et al., 2005). Coho and pink salmon show a strong tissue response to attached *L. salmonis* during the first 24 hrs (Wagner et al., 2008). This may explain why juvenile Coho salmon and pink salmon above 0.5 g of weight are less prone to sea lice infection and mortality than more susceptible species (Johnson & Albright, 1992; Jones et al., 2007; Nendick et al., 2011).

### 1.2.4. Transmission

Transmission of sea lice is mediated by its planktonic stages. The dispersal range of these larvae will depend mostly on water currents (McKibben & Hay 2004; Brooks, 2005; Costello 2006; Penston et al.,

2008; Amundrud & Murray, 2009; Krkosek et al., 2010; Molinet et al. 2011), although temperature and salinity impact this range as well, as these factors affect development and survival of the parasite (Genna et al., 2005; Bravo et al., 2008a).

Several studies have found that planktonic stages can be transported tens of kilometres from their source by currents (McKibben & Hay, 2004; Costello, 2006). More recently, an observational study by Kristoffersen et al. (2013) suggests that *C. rogercresseyi* can be transmitted up to 30 km. In intensive salmon farming regions like Chile, where farms are closely located, this implies there is transmission of sea lice among farms. This situation can lead to impaired treatment efficacy as the treated fish may be colonized shortly after treatment by new lice originating from neighbouring farms (SEARCH project, 2006). High transmission between farms also increases the risk of resistant gene exchange between sea lice populations (Tully & Nolan, 2002; Todd et al., 2004).

Transmission of sea lice between infected fish on the same farm is mediated to a great extent by host density. When the host density is high, the chances of sea lice exchange between fish are greater, as hosts are closer together; the increased transmission at this level increases the reproductive rate (Keeling & Rohani, 2007). Further, it has been described that, farms with a large number of fish contribute more to the density of copepodids in the water column than farms with fewer fish (Penston & Davies, 2009).

Copepodids can originate from infected farmed or wild fish. In the first case, the influx of copepodids to surrounding waters will be a function of the number of gravid females and their fecundity on farmed fish (Penston & Davies, 2009; Torrissen et al., 2013). Kristofersen et al. (2013) found a significant association between the number of gravid females at neighbouring farms and the level of juvenile lice on farms. Several studies on *L. salmonis* have linked wild fish migrations to an increased infestation pressure on farmed salmon, suggesting infected wild salmon may influence the copepodid concentration in a particular area (Jackson et al., 1997; Marshall, 2003). In Chile, *C. rogercresseyi* has been found on wild

native fish and on free-living salmonids close to salmon farms (Carvajal et al., 1998; Bravo, 2003), so it is possible that wild fish play a role in sea lice transmission, though explicitly testing for this factor is outside the scope of this thesis.

### 1.2.5. Impacts at farm and industry levels

Sea lice produce economic losses associated mainly with costs of treatments. Other costs are reduced product marketability, impaired growth and feed conversion efficacy, secondary infections and, in extreme cases, direct mortality (Costello, 2009). Economic impacts at the farm level have been estimated between US$ 0.18 and 0.45 per kg of produced salmon in Scotland, and between US$ 0.08 and 0.11 per kg of produced salmon in New Brunswick, Canada (Johnson et al, 2004). In Chile in the late 1990s, losses were estimated in US$ 0.3 per kg of produced salmon (Carvajal et al., 1998). In this study, the mean abundance was 4.9 louse per fish (Carvajal et al., 1998).

At the industry level, some of the impacts due to sea lice are negative publicity from the use of parasiticides that may leave residues in fish meat, and increased control required where farms may act as sources of sea lice that cause mass infestations (Costello, 2009).

Regional impacts of sea lice have been estimated at 4% of the production value in Atlantic Canada (Mustafa et al., 2001), and between 7 and 10% in Scotland (Rae, 2002). In a more global estimation of sea lice economic impacts, Costello (2009) calculated the sea lice costs to the world salmonid farming industry, for 2006, as $480 million USD, which was 6% of the worldwide production value that year.

### 1.2.6. Risk factors for sea lice infection

Observational and experimental studies have suggested a group of factors as determinants for sea lice abundance which can be broadly classified into environmental parameters and management practices. Among environmental factors, it has been shown that water temperature has a major effect on the

abundance of both *C. rogercresseyi* and *L. salmonis* (Brooks, 2005; Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Jansen et al 2012; Kristoffersen et al., 2013). Higher temperature accelerates the rate of development of all life stages, making the cycle is shortened and, therefore, producing more generations of lice in a given time (Johnson & Albright, 1991; Wadsworth et al., 1998; González & Carvajal, 2003). Furthermore, Tucker et al. (2000) observed that low temperatures reduce copepodid settlement success. It has also been described that the water temperature influences the survival of all life stages of *L. salmonis* and probably also it impacts survival (Stien et al., 2005).

Water salinity is another environmental factor that has been described as impacting positively of *C. rogercresseyi* and *L. salmonis* (Brooks, 2005; Genna et al., 2005; Bravo et al., 2008a; Yatabe et al., 2011; Kristoffersen et al., 2013). Research conducted in *L. salmonis* have found that the larvae do not consistently develop into the infectious stage at salinity levels below 25 ppm, affecting the settlement of copepodids on fish (Brooks, 2005; Genna et al., 2005; Costello, 2006; Bricknell et al., 2009). A study by Bravo et al. (2008a) revealed that water salinity levels also impact the survival of adult stages of *C. rogercresseyi*. A study from Kristoffersen et al. (2013) suggested that water salinity also impacts the development of *C. rogercresseyi* on host.

Mean fish weight is another host-related factor positively associated with sea lice abundance (Tucker et al., 2002; Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Jansen et al 2012; Kristoffersen et al., 2013). The fish weight effect on sea lice abundance is probably due to the fact that larger fish has been exposed longer time to sea lice. Saksida et al. (2007) described that fish age has a significant effect on the abundance of *L. salmonis* in Atlantic salmon.

The use of pharmacological treatments is another factor that, in general, has an impact on sea lice abundances at the farm level (Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013). Depending on the type of drug, treatments may tackle specific life stages of sea lice (see section 1.2.2),

which, in turn, may affect the parasite population composition and fitness. However, it has been demonstrated that impacts of chemotherapeutants in sea lice populations are temporary, and therefore, they should not be used as the only control strategy.

Finally, the external infectious pressure exerted by neighbouring farms is another factor that may affect sea lice abundance at the farm level. Jansen et al. (2012) measured the infectious pressure as the local biomass density in a 40 km radius from individual farms in Norway, and found a strong effect on sea lice levels and efforts to control infections. Another study carried out in Norway revealed that 28% of the in-farm sea lice level may be attributed to neighbouring farms (Aldrin et al., 2013). More recently, in a study aimed to evaluate the sources of sea lice in Chile, Kristoffersen et al. (2013) found the number of gravid females at neighbouring farms within 30 km seaway distance impacted significantly the juvenile *C. rogercresseyi* mean abundance at the farm level. These examples provide evidence the external source of lice must be considered as an important component of the sea lice abundance at the farm level.

### 1.3. Current control methods

Strategies for controlling sea lice levels in salmon aquaculture, used currently or in studies, are varied, and include management practices, biologic control, vaccination, selective breeding, used of immune-modulators, and chemotherapeutants (Jones, 2009; Burka et al., 2012; Torrissen et al., 2013). Whatever the control method used, in most salmon-producing regions, local authorities encourage the adoption of integrated pest management (IPM) programmes with the objective of applying control methods that impact sea lice levels through different mechanisms, and in this way, provide an integrated approach to sea lice control (Grist, 2002; Rosie & Singleton, 2002; Health Canada, 2003; Heuch et al., 2005; BC Ministry of Agriculture and Lands, 2008). This thesis was focused on the use of chemotherapeutants as a control measure for *C. rogercresseyi*.

### 1.3.1. Management practices

General good management practices for sea lice control on a farm should include fallowing between production cycles and single year-class stocking (Revie et al., 2009). These strategies are designed to interrupt the sea lice life cycle and minimize the transmission of sea lice between fish of different ages. In Chile, fallowing periods have been set to span three months and have to be done in a coordinated manner, meaning a particular neighbourhood has to have a minimum of three months where all farms are fallow. Fallowing periods are set every 21 to 24 months, where the last three months are fallowed. As the length of an Atlantic salmon production cycle is practically twice that of Coho salmon and rainbow trout cycles, in multi-species neighbourhoods there will be mixed year classes of rainbow trout and Atlantic salmon during the 24 month production period. This can pose a problem for controlling sea lice infections.

In addition, the farm should employ a monitoring program for sea lice counts and for resistance to antiparasitic agents, both with adequate sampling schemes and frequency (Revie et al., 2009). In Chile, sea lice monitoring is regulated by the Official Program of Surveillance and Control of Caligidosis, administered by the National Service of Aquaculture and Fisheries (Sernapesca). This program requires that salmon farms rearing Atlantic salmon or rainbow trout in operation report sea lice counts on a weekly basis, classified by juvenile, mobile adults (non-gravid females and male adults), and gravid females, for 40 fish sampled randomly from four cages (10 fish per cage) (Sernapesca, 2012).

Other husbandry practices can be deduced from results of risk factor studies, such as reducing the number of fish, the stocking density, and the use of less susceptible salmon species (Revie et al., 2003; Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013; Bravo et al., 2014). Site selection is another important factor for reducing the risk of infectious diseases due to proximity to neighbouring farms which is currently being considered in the settlement of new salmon farms in Chile (Alvial et al., 2012).

### 1.3.2. Biological control

This strategy consists of finding a natural predator of sea lice and stocking it in sea net pens along with farmed salmon to reduce lice burdens. Different species of wrasse have been used as cleaner fish in Norway and, to a lesser extent in Scotland and Ireland (Treasurer, 2002). There are no experiences with cleaner fish in other salmon-producing regions, such as Chile, as no indigenous fish that can fulfill this function have yet to be found (Torrissen et al., 2013). Wrasse is efficient in removing mobile sea lice stages from salmon, but have little effect on removing juvenile lice (Costello, 2006). A potential problem of using wrasse as a biological control is the possibility that this species act as a reservoir for salmon infectious pathogens (Treasurer, 2002).

### 1.3.3. Control methods under development

#### 1.3.3.1. Breeding Selection

Research has shown that the susceptibility of Atlantic salmon to sea lice is variable and it can be associated with specific fish families. Also, it has been reported that susceptibility is linked to major histocompatibility complex (MHC) Class II (Glover et al., 2007). Recent research has shown high resistant (HR) were associated to lower sea lice levels than low resistant (LR) Atlantic salmon families. In addition, the same study demonstrated that the expression of selected genes from skin tissue was lower in LR fish than in HR fish, which suggests the ability to resist lice infection depends on the ability to avoid immunosuppression (Holm et al., 2015).

#### 1.3.3.2. Vaccine

There have been attempts to find suitable vaccine antigens in *L. salmonis* and *C. rogercresseyi* in order to immunize Atlantic salmon, with relative success in controlled environments (Grayson et al., 1995; Carpio et al., 2011). This control strategy is still in the development stages.

#### 1.3.3.3. Immuno-modulators

Immuno-modulators act by enhancing systemic and localized inflammatory mechanisms through the stimulation of the fish immune system prior to exposure to sea lice; this accelerates and boosts fish response to sea lice leading to greater protection against infection (Burka et al., 2012). There are in-feed immuostimulant products available for the industry, but to date these have been used only in small-scale productions (Torrissen et al., 2013).

### 1.3.4. Pharmacologic treatments

Antiparasitic agents can be broadly classified in two groups, based on their administration method: in-feed additives or immersion treatments, the later also known as bath treatments. Avermectins and chitin synthesis inhibitors are administered as in-feed additives, while synthetic pyrethroids, organophosphate and oxidizing agents are delivered through bath treatments. Table 1.2 summarizes the current available pharmacologic options for control of sea lice.

#### 1.3.4.1. Avermectins

Avermectins are chemotherapeutants that open glutamate-gated chloride channels which increase chloride concentration and inhibit neural transmission (Grant, 2002). The avermectin currently used in controlling sea lice is Emamectin benzoate (EB), known commercially as SLICE®. Ivermectin was also used for this purpose in the past (Burridge, 2003), although it is used today in Eastern Canada as off-label use in the first year of production. EB targets all sea lice stages attached to the host, including adult and juvenile stages. Dosage is 50 μg/kg/d, for 7 consecutive days (Stone et al., 1999). When effective, EB generally provides an extended period of protection (Stone et al., 2000); in studies by Jones et al. (2012, 2013) sea lice levels remained at a low level up to 8 weeks after an EB treatment (years 2004 to 2006) in New Brunswick. However, due to its extensive use in practically all salmon-producing regions, populations of both *L. salmonis* and *C. rogercresseyi* have been associated with lower sensitivity to EB (Lees et al., 2008a; Bravo et al., 2008b; Jones et al., 2012, 2013). Based on studies by Jones et al. (2012, 2013), EB

had little effect in New Brunswick after 2008, both on the sea lice drop and the duration of the effect.

#### 1.3.4.2. Organophosphates

Several organophosphate drugs have been used to control sea lice in the past, including Malathion, trichlorfon, and dichlorvos. Today, the only organophosphate approved is Azamethiphos (Salmosan® in Canada and Europe; Byelice® in Chile) (Burridge et al., 2010). This compound is administered through baths at a dose of 100 μg/L, for 30 to 60 minutes, and exerts its action by inhibiting the acetylcholinesterase (AChE) activity (Kazemi et al., 2012). It is effective in killing adult and pre-adult stages of sea lice, but less effective when targeted at juvenile lice (Roth et al., 1996). Azamethiphos is registered for use in Norway, Chile, and Scotland, and it has an emergency registration status in Canada (Burridge et al., 2010; Burridge et al., 2011). Clinical trials performed in Scotland have shown that the efficacy of azamethiphos on pre-adult and adult *L. salmonis* was at least 85% (Roth et al., 1996).

#### 1.3.4.3. Chitin synthesis inhibitors

These compounds inhibit chitin synthesis in the copepod exoskeleton and, consequently, these are targeted at larval and pre-adult stages (Torrissen et al., 2013). Two drugs have been registered for use in controlling sea lice chitin synthesis, namely diflubenzuron (Lepsidon®) and teflubenzuron (Calicide®). Chitin synthesis inhibitors have been used in Scotland and Chile, at a dosage of 10mg/kg/d, by 7 days, and it has also been manufactured for the Canadian market (Burridge et al., 2010). Chitin synthesis inhibitors are not the farmer's first drug of choice in Chile, but, when used, they are administered along with other drugs, such as pyrethroids, that target adult stages (R. Ibarra, pers. comm.). Clinical trials in Scotland and Norway found the efficacy of teflubenzuron (Calicide®) on total sea lice was around 85% (Branson et al., 2000).

#### 1.3.4.4. Oxidizing agents

The most common oxidizing agent used to combat sea lice is hydrogen peroxide. It has been suggested

that this compound produces a mechanical paralysis due to bubble formation in the gut and heamolymph, resulting in detachment from the host (Bruno & Raynard, 1994). Hydrogen peroxide is primarily targeted at mobile stages, although it appears to have some efficacy on juvenile lice (Bravo et al., 2010). It is applied at a dosage of 0.5 g/L, for 20 min, through immersion treatment (Treasurer et al., 2000). In a field trial, its efficacy on adult *C. rogercresseyi* averaged 70% (Bravo et al., 2010). Hydrogen peroxide is authorized in all salmon-producing countries, but as detached parasites can recover from the treatment and infest a new host (Bravo et al., 2010), this drug, in general, is not a treatment of choice.

#### 1.3.4.5. Synthetic pyrethroids

Synthetic pyrethroids for controlling sea lice include cypermethrin (Exis®), high-*cis*-cypermethrin (Betamax®) and deltamethrin (Alphamax®). Pyrethroids interfere with nerve impulse transmission by stimulating the sodium channels in neural cells that produce spastic paralysis and subsequently death of the parasite (Burka et al., 2012; Torrissen et al., 2013). These products appeared in the market in the mid-1990s and replaced the organophosphates that were widely used at that time, due to their better performance and wider safety margin (Torrissen et al., 2013). Before 2005, Exis® was authorized in Scotland and Ireland, while Betamax® and Alphamax® were approved for use in Norway (Burridge et al., 2010). Later, Alphamax® was made available in New Brunswick, Canada, under emergency conditions (New Brunswick Agriculture and Aquaculture, 2011). In Chile, Alphamax® and Betamax® were authorized in 2007 and 2010, respectively (SAG, 2013), and quickly dominated the market as an alternative drug with the rise of resistance to EB.

In general, synthetic pyrethroids are targeted at mobile stages of sea lice, although Betamax® is authorized in Chile for juveniles as well (SAG, 2013). The recommended dosage for pyrethroid treatments ranges between 2.0 and 15 µg/L for 30 to 60 minutes, depending on the product (SAG, 2013). Low sensitivity of sea lice populations towards synthetic pyrethroids has been described in Norway, Ireland, Scotland, and Chile through bioassay (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005a;

Helgesen et al., 2014). In general these products have relatively good field efficacy on adult *C. rogercresseyi* ranging from 70 and 99% (Bravo et al., 2013, 2014a).

### 1.3.5. Use of antiparasitic drugs in Chile

At the present, five drugs are approved for use against *C. rogercresseyi* in Chile, namely emamectin benzoate, deltramethrin, cypermethrin, diflubenzuron, and azamethiphos (SAG, 2013). Although there are not official information regarding the use of chemotherapeutants for controlling sea lice in Chile, it has been estimated that azamethiphos is the most widely drug used in 2014 with 60% of treatments approximately. Pyrethroids account for 30% while emamectin benzoate only for 10% of treatments or even less (Rolando Ibarra, SalmonChile, pers. comm.). Combination of drugs with different lice stage targets (i.e. pyrethroid and emamectin benzoate) in a single treatment may occur, especially during the first half of the production cycle when there is no risk of exceeding the withdrawal period, although that is not a very common practice (R. Ibarra, SalmonChile, pers. comm.).

### 1.3.6. Therapeutant administration methods

#### 1.3.6.1. In-feed treatment

In-feed treatments are easier to deliver to the fish than immersion treatments. A key advantage is that, in general, oral drugs remain longer in the fish's body, which provides a longer protective effect, than drugs administered through baths. However, when dealing with diseased or stressed fish, some individuals do not feed, and therefore, will receive sub-therapeutic dosages. Critical points in medicated feed formulation are a proper coating of the pellet with the drug solution, and an appropriate storage that minimizes lixiviation of the drug (Burka et al., 2012). Another disadvantage of in-feed treatments is they have a longer withdrawal period than topical products (e.g. pyrethroids) (San Martín et al., 2010). For this reason, in-feed treatments are generally avoided in fish close to the market weight (R. Ibarra, pers. comm.). The duration of oral treatments is, in general, 7 days (SAG, 2013) and, because oral treatment at every cage can be performed simultaneously, the farm-level treatment should also last 7 days.

#### 1.3.6.2. Immersion treatment

In bath treatments, the drug is delivered to fish by pouring a drug solution into the fish pen or in a well boat. When the treatment is performed in the fish pen, this method requires the temporary placement of either a skirt or a tarpaulin around the cage in order to minimize the dilution or loss of the drug inside the pen by the ocean currents (Burka et al., 2012). Skirts are placed around the fish net wall, while a tarpaulin covers the bottom of the net as well as the side walls. There is no evidence in the literature that suggests differences in treatment efficacy between tarpaulins and skirts. However, in Chile, the regulations have recently removed the skirt as an option for immersion treatment (Sernapesca, 2012). Whether tarpaulin or skirt is used, it is also necessary to reduce the volume of water, so the net needs to be raised, which stresses the fish. As a consequence, manufacturers strongly recommend oxygenation of the water during the procedure. Because the water volume is not precisely controlled by the manager, the drug concentration is not guaranteed. In an experimental study it was determined that the rate of retention of the drug in skirted cages depends, in part, on the current speed beneath the cage, and the ratio between the skirt and the net depth (Corner et al., 2011).

Bath treatment of one cage using a skirt or tarpauline usually takes between 2 to 5 hours, depending on the weather conditions (R. Ibarra, pers. comm.). An important drawback of immersion treatment is that re-infestation after the procedure can occur as the drugs do not have long lasting effects on the fish. Lice that colonized recently treated fish can originate from neighbouring farms (Jansen et al., 2012; Kristoffersen et al., 2013) and/or form the farm itself (Costello, 2006; Kristoffersen et al., 2013). The latter situation is known as self-infestation and occurs because immersion treatments are demanding in manpower and equipment, so they cannot be performed simultaneously in all cages on a farm (contrary to in-feed treatments). Fish from first treated cages can be infected by fish from cages that have not been treated yet (Costello, 2006). One positive aspect of immersion treatments is that, if the drug mixing at the cage is properly done, all fish are treated equally (i.e. the same dose), in contrast with in-feed treatment. Another advantage of bath treatments is that the withdrawal period for topical drugs is substantially shorter than

for in-feed drugs, as the drug is not incorporated in the fish tissues (San Martín et al., 2010). For example, withdrawal period for drugs administered through bath treatments (deltamethrin, cypermethrin and azamethiphos) ranges from 10 to 30 degree-days (1 to 3 days when water temperature is 10° C), while for diflubenzuron (oral drug), withdrawal period is 300 degree-days (~ 30 days at 10° C) (SAG, 2013).

Well-boats are vessels designed for the transport of live fish. Because well-boats fully control water flow, oxygen and carbon dioxide, they have become a practical alternative to tarpaulins or skirts for immersion treatments. In addition, well-boats can dispose treated water off-farm reducing environmental impacts. On the other hand, well-boats are also more expensive to operate and are not always available.

### 1.3.7. Treatment strategies

Guidelines for treatment strategies are established by both the government and farmer associations in order to optimize the use of pharmacologic treatments. Main strategies include: 1) setting a treatment trigger threshold, and 2) treatment coordination.

#### 1.3.7.1. Trigger threshold (action level, trigger level)

The action level is a sea lice abundance per fish above which farms are mandated to apply control measures, which in general comprise the initiation of a delousing treatment. In some countries, such as Chile, the control measure may be the anticipated harvest of fish (Sernapesca, 2012). Trigger levels are changing over time in all salmon-farming regions as management programs evolve and new control methods are developed.

In some countries, the action level setting takes into account the time of the wild salmon migration in order to mitigate the risk to wild stocks. This has been the case in British Columbia, Canada since 2004, where the trigger threshold was 3 mobile *L. salmonis* per fish during the wild salmon migration period (March to June) and 6 for the rest of the year. More recently, the action level in that region is 3 mobile *L.*

*salmonis* per fish year round (Saksida et al, 2007); however, increased monitoring frequency is required at certain times of the year (treatments or harvesting). Similarly, before 2008, the action level in Norway was set at 0.5 adult females or 5 total mobile lice per fish between December 1st and July 1st, while the rest of the year the limit was 2 adult females or 10 total mobile lice per fish. Under the current regulations in Norway, treatments have to be performed with more than 0.5 adult females or 3 total mobile lice per fish during the entire year (Ritchie & Boxaspen, 2011).

In Ireland, originally an action level was set only during spring time (March-May), at 2 ovigerous lice per fish. In a later modification, the management program considered the reduced sea lice levels in spring time and lowered the trigger level to 0.5 ovigerous lice per fish, and 2 for the rest of the year (Revie et al., 2009; Jackson, 2011). In Scotland, the aquaculture industry has set the trigger threshold at 0.5 adult female per fish during the wild smolt migration period (February-June), and 1 adult female for the period between July and January (Revie et al., 2009).

Before 2012, the Chilean authority had set trigger thresholds for mandatory and voluntary delousing treatments, taking in consideration the lice development stage. For example, compulsory treatments were required with 6 or more total adult lice, or with 6 or more juvenile lice. Within the ranges of 3 to 6 adult lice or 1 to 6 juvenile lice, treatments were voluntary; and with fewer than 3 adult lice or 1 juvenile lice, treatments were not permitted. In addition, the regulation stated the authority was allowed to reduce the action level based on technical criteria, such as historic sea lice burdens, current fish biomass, and the presence of holding facilities in the area (Sernapesca, 2009). In the current regulation, a mandatory trigger threshold is set at 6 or more total adults per fish only for farms located within a five nautical mile radius of a high dissemination farm (9 or more total adults per fish). For farms with fewer than 6 total adults per fish the delousing treatment is voluntary (Sernapesca, 2012).

#### 1.3.7.2. Treatment coordination

Coordinated treatments are procedures where a group of spatially related farms are encouraged to perform delousing treatments within a predefined time. Although the general aim of coordinated treatments is to reduce the sea lice levels in an area, these procedures may have more specific objectives depending on the local conditions. For example, in Norway, treatment coordination is primarily aimed to reduce the risk that farmed salmon contribute sea lice to migrating wild salmon populations. These procedures are also called "strategic" coordinated treatments.

In contrast, where wild fish are not part of the sea lice control strategies, such as in Chile, treatment coordination is targeted to improve treatment efficacy. In this setting, the rationale is to interrupt the sea lice life cycle in a defined area at the same time, and in that way, reduce the re-infestation after treatment. Given that treatments need to be carried out in a relative short period of time, in these cases, the term "synchronized" is a better descriptor of the activity than "coordinated".

**Strategic coordinated treatments**

Strategic coordinated treatments (SCTs) have been put into practice in most of salmon-producing countries of the Northern Hemisphere. In general, coordination of treatments are performed one or two times per year and are aimed to keep sea lice levels lower in time and thus minimize the impact on farmed and wild fish (Rae, 1999; Jackson, 2011; Revie, 2011; Ritchie & Boxaspen, 2011; Saksida et al., 2011). As an example, the winter strategic coordinated treatment carried out in Norway is aimed to eliminate overwintering female lice which are responsible for the spring peak of juvenile lice as water temperature rise (Ritchie & Boxaspen, 2011). In turn, the spring strategic coordinated treatment is aimed to kill the newer cohort of lice. Winter strategic coordinated treatments in BC are intended to decrease the sea lice level during spring when migrating pink salmon move into the coast. Consequently, the treatment trigger threshold is relatively low (Saksida et al., 2011).

It has been reported that SCTs have beneficial effects on sea lice management. For example, Wadsworth (1998) attributed a significant reduction of adult lice, fish mortality, fish downgrading at harvest and number of anti-lice treatments between two time periods to winter strategic coordinated treatment procedures in Scotland. Later, based on an analysis of sea lice trends in time it was suggested that sea lice burdens decreased as a consequence of the implementation of annual spring strategic coordinated treatment (Revie, 2011). Similar decreasing trends have been reported in Norway after running winter SCTs for several years (Ritchie & Boxaspen, 2011). On the contrary, in a multivariable analysis where management and environment factors were taken into account, it was concluded that SCTs did not have a significant effect on the sea lice levels (Revie et al., 2003); however, it was later suggested that this potential association might be affected by confounding (Revie, 2011). The strategic coordinated treatment applied in BC before the migration of juvenile pink and chum salmon in the Broughton Archipelago has coincided with a significant and persistent decline in *L. salmonis* abundance on the wild salmon (Jones & Hargreaves 2009).

**Synchronized treatments**

In Chile, the legislation in force at the time of this study encouraged treatment synchronization year round, by means of setting temporal windows in which farmers carry out delousing treatments. In general, these windows last 7 days and are repeated every two weeks throughout the year. However, because this legislation did not consider a mandatory treatment trigger threshold, the treatment synchronization remained as voluntary. Only in the presence of "spreader farms" (farms with $\geq 9$ total adult lice/fish), the treatment synchronization is mandatory within a 5 nautical mile radius area from the affected spreader farm, but only for those neighbouring farms with 6 or more total adults per fish (Sernapesca, 2012). The current legislation has changed the definition for "spreader farm" to farms with $\geq 3$ gravid female lice/fish, but no specifications are made in relation to the trigger threshold for neighbouring farms (Sernapesca, 2015). There is no published information regarding the evaluation of synchronized treatments in Chile.

From a practical point of view, synchronization of immersion treatments is challenging because the time required to treat entire farms may vary considerably. The duration for treating a cage is 2 to 3 hours, and due to immersion treatments being highly demanding in personnel and equipment, generally only one cage can be treated at a time. Therefore, it takes approximately 7 days to treat all cages in an average farm (approximately 20 cages). The speed at which the farm personnel treats a cage varies as well, and can be negatively impacted by weather conditions.

### 1.3.8. Treatment success evaluation

Treatment success is evaluated by comparing the sea lice levels before and after the treatment. This is also called treatment efficacy or effectiveness. In practice, treatment efficacy has been expressed as percent reduction ([pre-treatment – post-treatment] / pre-treatment) (Branson et al., 2000; Gustafson et al., 2006). A similar way to calculate efficacy is to express the post-treatment level as ratio of the pre-treatment level (post-treatment / pre-treatment) (Jones et al., 2012, 2013). Other studies have expressed the treatment efficacy by modeling the post-treatment level with a regression model, while accounting for the pre-treatment level by including it in the model as a predictor (Lees et al., 2008a).

In general, the pre-treatment measurement is taken as close as possible before the procedure, while the post-treatment level is assessed over a wider range of time after the event, depending on the drug used. Topical treatments are commonly evaluated between 3 and 7 days after treatment, while oral treatments are evaluated as early as 1 week after treatment and up to, for example 12 weeks, in studies aimed to evaluate treatment success over time (Jones et al., 2012).

#### 1.3.8.1. Variability in counting of sea lice

An aspect that needs to be considered in the evaluation of treatment efficacy is the variability of counts between the pre-treatment and the post-treatment sample. This variability has, at least, two components. First, the random variability which is present among sea lice samples because a different set of fish is

sampled from the same population. Random variability can be addressed for by hypothesis testing using appropriate sample statistics. The second main source of variability is due to differences in precision of counts between counters, which can be classified as a measurement error. This type of error cannot be accounted for unless information of counters is included in the analysis. A recent study conducted in *L. salmonis* in New Brunswick found significant differences between lice counters, especially when loads of juveniles, pre-adult and adult males were high (Elmoslemany et al., 2013).

#### 1.3.8.2. Assessing the treatment success with multivariable techniques

In an ideal situation, treatment events should be evaluated in identical conditions to make the values comparable. However, in practice, delousing treatments are performed in different environmental and management conditions. For example, in few cases, pre- and post-treatment samples are taken at fixed times relative to the treatment onset and end, but mostly they are taken as part of the regular monitoring schedule. This situation makes the time between the pre- and post-treatment measurements variable from one event to another, and it is well known that premature evaluation of a treatment may impact its outcome (SEARCH project, 2006). Other factors that may affect treatment outcomes are that treatments are performed at different times of the year (i.e. seasons) and in different geographical areas and with varying fish health conditions (Lees et al., 2008a; Jones et al., 2012).

A relatively new approach to dealing with variability in the evaluation of delousing treatments is the application of multivariable techniques which allow for control of different sources of variation and, in that way, make treatment results more comparable. Multivariable delousing treatment evaluations, in this case EB treatments, have been published by Lees et al. (2008a), Jones et al. (2012, 2013).

### 1.3.9. Treatment failures

In general, the concept of treatment failure refers to a situation where the outcome of a delousing treatment does not meet the expected reduction of sea lice levels. Treatment failure is strictly associated

with clinical evaluations of field results. One concern raised by a failed treatment is the potential for reduced sensitivity of the sea lice to the particular antiparasitic agent. However, other causes may also influence the treatment outcome (see next sections), so it is very important to rule out treatment errors as a cause of failure.

Treatment failures have been reported in most of the salmon-producing countries in association with different drugs. For example, in the Northern Hemisphere failed treatments with pyrethroids have been documented in Norway, Ireland, and Scotland (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005a). Treatment failures ascribed to resistance to the organophosphates dichlorvos and azamethiphos have been reported in Norway and Scotland from mid-1990's (Denholm et al., 2002; Fallang et al., 2004). In the case of oral treatments, failed EB treatments have been reported in Scotland and eastern Canada (Lees et al., 2008a, b; Jones, 2012, 2013). In Chile, the situation is no different, as treatment failures associated with EB and pyrethroids have been observed since 2005 and 2008, respectively (Bravo et al., 2008b; Bravo et al., 2013).

#### 1.3.9.1. Common causes of treatment failures

The origins of treatment failures can be broadly classified as problems with drug administration, re-infestation after the procedure, and low sensitivity of the sea lice strain to the particular antiparasitic agent.

**Drug administration**

Problems with the drug administration may impair the delousing treatment outcome, especially when the drug is administered as a bath treatment, because this procedure involves some operational challenges (see section 1.3.5.2). Some common problems associated with bath treatments include incorrect calculation of the water volume, insufficient mixture of the agent in the netpen, insufficient oxygenation of the netpen during treatment, insufficient reduction of pen-volume during treatment (fish net lifting),

shortened exposure time of fish to drug solution, improper use of tarpaulin or skirts resulting in a leakage of the treatment solution, and low water temperatures during the procedure (SEARCH project, 2006).

Some pitfalls that may occur in oral treatments are errors in dosage rate as a result of erroneous calculation of biomass, premature termination of the treatment, splitting of the daily dose over several meals instead of administration as a single meal (SEARCH project, 2006). Incorrect dosage may also result from incorrect mixing of the feed pellet with the drug solution.

Another mistake that can occur with either bath or oral treatments is improper evaluation of the treatment result especially with regards to the timing of the assessment. A premature evaluation may result in higher sea lice counts as, in some cases, the drug effect does not occur immediately. On the other hand, a late treatment evaluation could result in high sea lice levels as a consequence of re-infestation from the farm itself or neighbouring farms.

**Re-infestation**

In general, re-infestation refers to the colonization by new sea lice that originate in neighbouring farms after a treatment event. Re-infestation may significantly impair the immersion treatment results in areas with intense salmon farming activity (SEARCH project, 2006). In general, the longer the time between treatment and post treatment sea lice assessment the higher the lice count, because more time has elapsed for re-infection and lice development. Based on developmental times for *C. rogercresseyi* described by Bravo (2010), and assuming a water temperature of 11° C, it is possible to predict that new chalimii would be observed as soon as 6 days after the treatment, while adult lice would be evident at 22 days after the procedure.

A recent study identified different sources of sea lice in Chile and determined that the infection pressure from neighbouring farms was greater than that coming from within the farm itself (Kristoffersen et al. 2013). The increasing usage of immersion treatments, which do not provide long lasting protection,

makes re-infestation an important issue.

Self-infestation is another source that should be considered in treatment performance evaluation. One common situation is when treatment of the whole farm takes too long, and lice can re-infest fish previously treated in other cages (Costello, 2006). Another situation is when a treatment targets only adult lice, and surviving juveniles have developed into adult lice by the time the post-treatment sample is taken. The time in which juvenile lice evolve into adult depends on the water temperature (Bravo, 2010). For example, at 8° C *C. rogercresseyi* chalimus III that have survived a treatment will develop into adults in 8 days, while at 13° C this time is reduced to 5 days.

**Resistance**

Resistance has been defined as the existence of a strain capable of surviving a dose of a control agent which is lethal to the vast majority of individuals in a normal population (ffrench-Constant et al, 2004). A pest population becomes resistant when the allelles conferring this ability to survive a particular chemotherapeutant dominate in the pest population over "sensitive" allelles. This shift in the gene composition is driven by selection, which happens when the population is exposed to the agent. The rate at which resistance develops in a population depends on many inter-related factors, including the resistance mechanism, the biology of the pest, existence of refugia, and the frequency of chemical usage (Denholm et al. 2002). Another important factor that promotes resistance is a limited diversity of compounds available for inclusion in control strategies, leading to over-reliance on single products or chemical classes (SEARCH project, 2006).

Resistance mechanisms are not fully described in sea lice; however, it is believed that, as with agricultural pests, the two most likely mechanisms are an increased ability to detoxify the chemical and a structural alteration to its target site. Detoxification can be caused by enhanced oxidative breakdown by enzymes known as mixed function oxidases (MFOs), enhanced activity of glutathione-S-transferases (GSTs),

which is an important mechanism in organophosphate resistance, and enhanced esterase activity that breaks down ester bonds, common in organophosphates and pyrethroids (SEARCH project, 2006). Evidence for the occurrence of the detoxification mechanism in sea lice has been provided by Sevatdal et al. (2005b), who found specific monooxygenases involved in detoxification of pyrethroids in *L. salmonis*.

Organophosphates exert action by inhibiting AChE, which is a key enzyme in the nerve impulse transmission of lice, while pyrethroids act by blocking ion channels in nerve cell that are also vital for the transmission of nerve impulses. In a study by Fallang et al. (2004), the presence of two AChE enzymes with different sensitivities towards azamethiphos was observed, confirming the target-site resistance mechanism in *L. salmonis* isolated from Norway and Canada. In another study by Fallang et al. (2005), researchers found that resistance to pyrethroids in *L. salmonis* from Norway was caused by point mutations in the sodium channels, suggesting a structural alteration of the target site as the mechanism for resistance to pyrethroids. In a more recent study, Kaur et al. (2015) demonstrated that resistance of *L. salmonis* to azamethiphos is due to a mutation in one AChE gene.

Another mechanism of resistance described in parasites is the enhanced elimination of the agent, mediated by P-glycoprotein efflux pumps (Tribble et al., 2007). Recent studies have provided some evidence that this resistance mechanism may be responsible for the low sensitivity of strains of *L. salmonis* to EB (Heumann et al., 2012; Igboeli et al., 2012).

*Methods for estimating resistance in sea lice populations*

Confirmation of resistance development is normally through the identification of biochemical or biophysical properties known to convey an advantage to parasites which can be transferred genetically to offspring (Jones et al., 2013; SEARCH project, 2006). If the mechanism of resistance is known, the most cost-effective method is to study the expression of this mechanism, e.g. the activity of detoxifying enzymes, or the presence of mutations associated with resistance (i.e. quantitative PCR-based assay).

These diagnostic methods can normally only be carried out in a laboratory environment. If the mechanism is unknown, the bioassay is the recommended diagnostic tool because it detects the response of sea lice to the antiparasitic agent in the field (SEARCH project, 2006). If this response is diminished and other causes of low treatment efficacy have been ruled out, the sea lice may have a low sensitivity to the drug.

In general, bioassays consist of collecting sea lice from infested fish, infecting salmon under controlled conditions (i.e. tanks), and challenging the offspring of these lice (usually pre-adult stages) with an antiparasitic drug. It is important to record the treatment performance history on the farm where the sea lice originate, specifically whether or not there have been treatment failures. In order to compare sensitivities from different farms, it is necessary to choose a "negative control," which can be sea lice from farms with no previous history of antiparasitic treatments. Sensitivity is determined as the concentration of drug at which 50% of target organisms are immobilized (i.e. moribund and dead lice), known as $EC_{50}$ (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005a).

Bioassays are expensive and require long-term monitoring of populations in order to detect differences over time. They also need large numbers of viable sea lice, due to the inherent natural variability in the gender and stage response to chemotherapeutants. In addition, the analysis is complex, as bioassays use different concentrations of the drug in order to determine the exact sensitivity in the tested population. Recently, Whyte et al. (2013) have proposed a modified bioassay that uses a fixed-dose approach, instead of the traditional dose-response bioassay.

Reports of treatment failures are another source of information for identifying reduced sensitivity which use routine monitoring data to compare sea lice levels before and after treatments. Results of these analyses must be taken with caution because treatment failures can be caused by a number of reasons (see section 1.3.8.1). There are different methods for reporting treatment failures based on field data, each of them providing different levels of evidence for resistance. These methods can be classified according to

Dohoo et al. (2009) as case reports and case series, surveys, experimental and observational studies. The first consists of describing the outcome of a treatment or a series of treatments selected by convenience. In the second (surveys), a greater number of treatments are included in a more formal study design Observational studies aim to explain the treatment outcome and control for extraneous variables with multivariable techniques. According to this classification, bioassays would fall in the experimental studies category.

*Evidence for resistance*

Evidence for resistance of sea lice to chemotherapeutants is abundant around the world (see Table 1.3). The first incidence of this phenomenon was reported in Norway, where tolerance to organophosphates, in particular azamethiphos, increased to the point of having totally lost their effect by mid 1990s (Jones et al., 1992; Roth et al., 1996; Fallang et al. 2004). Later, treatment failures associated with pyrethroids were reported in Norway, Scotland, and Ireland (Sevatdal et al., 2005a). Subsequent analysis, based on bioassay, confirmed reduced sensitivity to deltamethrin and cypermethrin (Sevatdal et al., 2005a). A recent study based on bioassays confirmed the low sensitivity of *C. rogercresseyi* to pyrethroids (Helgesen et al., 2014). Furthermore, this investigation found the level of sensitivity of the parasite in Chile to be practically equal to that of deltamethrin-resistant lice in Norway which constitutes strong evidence that pyrethroid-specific resistance genes are present in the Chilean sea lice genetic pool.

Reduced treatment efficacy associated with EB was found initially in Scotland (Lees et al., 2008a, b). At the same time, Bravo et al. (2008b) reported for the first time reduced sensitivity of *C. rogercresseyi* to EB in Chile using bioassays. Reduced efficacy and reduced sensitivity have also been reported in New Brunswick (Westcott et al., 2008; Jones et al., 2012, 2013; Whyte et al., 2013). The only exception for this trend of increasing tolerance of sea lice to EB is British Columbia where field and bioassay data suggest that resistance of *L. salmonis* to EB is not currently a problem (Saksida et al., 2010, 2013).

*Spatial variation of resistance*

The exchange of resistance genes between populations is a key element that explains resistance spread in space. A key aspect of sea lice biology with high impact on resistance development is the ability of lice to disperse and move genes over different spatial scales, a feature known as gene flow. Sea lice are able to do this when larval stages disperse and reach other sea lice populations or when the wild fish move attached lice. In general, when gene flow occurs between populations, the genetic structure is expected to be homogenous rather than structured; therefore, in sea lice, the genetic structure of the population is determined by the level of connectivity between patches of sedentary adults at the fish farms which depends on the mechanisms that governs the spatial and temporal pattern of distribution of the larval stages (Denholm et al. 2002; Nolan & Powell, 2009).

Research addressing spatial and temporal genetic variability of *L. salmonis* in the northern Atlantic has found, in general, a non-significant or weak genetic structure among sea lice populations (Todd et al., 2004; Tjensvoll et al., 2006; Nolan & Powell, 2009; Glover et al., 2011), which indicates an active exchange of genes within that region. In a recent study with the objective of evaluating the resistance dispersal and evolutionary connectivity among *L. salmonis* in Northern Atlantic, it was suggested that emamectin benzoate resistance developed at a single source, and spread across the Atlantic in approximately 10 years (Besnier et al., 2014).

Resistance studies based on bioassays, in which certain sea lice populations are exposed to particular antiparasitic agents, have revealed that low sensitivity varies across space on a more local scale (e.g. between farms). In general, these differences are either very small or non-significant (Sevatdal et al., 2005; Bravo et al., 2008b; Westcott et al., 2008) which suggests the flow of resistance-specific genes between farms. Conversely, other studies have observed a highly structured spatial sensitivity (Whyte et al., 2013), and resistance has also been described as varying across years and seasons (Bravo et al., 2008b; Westcott et al., 2008; Whyte et al., 2013).

Another source of information for the spatial and temporal variation of sea lice sensitivity to treatments is the efficacy based on field data. Studies on EB efficacy in Scotland and New Brunswick (NB), Canada, have shown differences across geographical regions (Lees et al., 2008a, b; Jones et al., 2012). Some of these studies have shown diminished treatment efficacy values as fish age (Lees et al., 2008a; Jones et al., 2012; Saksida et al., 2013), which could mean that resistance increases over the duration of a production cycle.

## 1.4. Current investigation

### 1.4.1. The problem

Infections caused by sea lice are a problem for the economic and environmental sustainability of salmon farming worldwide and controlling this pest is one of the industry's most significant challenges.

The main method for controlling sea lice is the use of chemotherapeutants, synthetic pyrethroids being the most used at present. The intensive use of synthetic pyrethroids has led to the development of resistance in some strains of *L. salmonis* in European countries (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005). Prior to this research, the only background information available in Chile were a number of anecdotal reports of low efficacy of treatments associated with pyrethroids. In many cases, Chilean farmers use synthetic pyrethroids as the only drug for control of sea lice and there are doubts about its effectiveness against juvenile stages of *C. rogercresseyi*. Recently, low sensitivity of *C. rogercresseyi* to deltamethrin has been reported in Chile (Helgesen et al., 2014). Greater insight into the susceptibility of particular lice development stages to specific drugs is needed, because it will ensure a better selection of products, and allow the application of targeted therapies, which may delay the development of resistance to chemotherapeutants.

It is well known that sensitivity of lice to antiparasitic agents has a genetic basis and that the exchange of these resistance genes can be very intense in some areas. Studies of genetic variability of *L. salmonis* have

revealed a population genetically unstructured (Todd et al., 2004; Tjensvoll et al., 2006; Nolan & Powell, 2009; Glover et al., 2011), which is in agreement with the gene flow hypothesis. In relation to that, it is essential to monitor the distribution of the sensitivity of lice to antiparasitic agents and particularly pyrethroids in time and space. Currently, there are no published studies that have addressed the spatial distribution of sea lice sensitivity to synthetic pyrethroids.

Furthermore, because pyrethroids are administered as topical drug, they do not confer a residual effect that is able to protect fish over a long period of time after the procedure. As a result, treated fish are quickly recolonized with new lice from the farm itself and neighbouring farms, worsening the apparent outcome of treatments. This situation is more pronounced in areas with intensive salmon farming where transmission of copepods between farms is high. For this reason, it is necessary to explore strategies that reduce re-infestation after the treatment for farms sharing the same body of water. Synchronization of treatments is a promising option; however, it has not been yet formally evaluated in any salmon-producing region. The Chilean context, which involves monthly, voluntary, synchronized treatments, weekly sea lice monitoring, and a large number of fish farms, offers a unique opportunity to evaluate treatment synchronization at the farm level, while controlling for external sources of lice and factors that affect the sea lice abundance at the farm itself.

Finally, it is important to mention that in most of the salmon farming regions, there are comprehensive monitoring systems that collect considerable amounts of information on sea lice levels, delousing treatments, management and environmental factors at the farm or even at the pen level, with a high sampling frequency in time. This research is intended to demonstrate how field data can be analyzed, following an epidemiological approach, in order to test hypotheses under field conditions.

### 1.4.2. General objective

The general objective of this research was to provide evidence for optimizing the use of synthetic

pyrethroids for controlling *C. rogercresseyi* in Chile, through the analysis of information generated by monitoring systems, using an epidemiological approach.

### 1.4.3. Specific objectives

#### 1.4.3.1. Study 1

Assess the performance of pyrethroid-based immersion treatments in Chile, between October 2011 and May 2012, and compare the effects of the three available products (Alphamax®, Betamax® and a generic deltamethrin) on the different development stages of sea lice.

#### 1.4.3.2. Study 2

Evaluate the spatial distribution of the performance of pyrethroid-based treatments, in Chile, between January 2012 and September 2013.

#### 1.4.3.3. Study 3

Evaluate the effect of synchronized treatments on sea lice levels at the farm level for a period of several weeks after the treatment event, from January 2012 to September 2013, in Chile.

### 1.4.4. Dataset for the current investigation

All the data utilized in this thesis were obtained from the Chilean salmon industry‘s Sea Lice Monitoring Program, administered by the Instituto Tecnológico del Salmon (Intesal). This monitoring program collects information for approximately 90% of the salmon farms in Chile located in the three main growing-up regions, namely Los Lagos, Aysén and Magallanes. Los Lagos and Aysén regions contain more than 93% of salmon farms that reported data to the SalmonChile Sea Lice Monitoring Program in 2012 and 2013. The study period for this thesis was October 2011 to September 2013. This study only included farms from Los Lagos and Aysén regions. During that time frame 529 farms located in 56 neighbourhoods from Los Lagos and Aysén regions reported sea lice data into the Program. These farms

belonged to 23 companies and reared Atlantic salmon (n=321), rainbow trout (n=129), Coho salmon (n=74) and Chinook salmon (n=5). Only farms rearing Atlantic salmon and rainbow trout were considered in the analyses of this thesis because of their susceptibility to sea lice. The mean number of fish stocked per farm during the study period was around 940 thousands fish (sd=240,000), with farms stocking as low as 116,000 to a maximum of 1.93 million fish. The maximum fish biomass during the farm's production cycle averaged 2,740 tons (sd=1,356) with minimum of 88 tons and maximum of 10,280 tons. Figures 1.1 and 1.2 show the total number of fish and fish biomass of Atlantic salmon and rainbow trout in Los Lagos and Aysén regions during the study period.

Farms participating in the SalmonChile Sea Lice Monitoring Program reports *C. rogercresseyi* counts of juvenile (mainly chalimus III and IV), mobile adults (male and non-gravid females) and gravid female lice, from a 10-fish sample each drawn from four pens on a weekly basis (40 fish in total). Sampling follows the protocols of the Official Caligidosis Surveillance and Control Program (Sernapesca, 2012) (see Appendix in this chapter for more details on sea lice sampling protocols). Farms also have to report delousing treatments information such as the drug used, and the dates of the procedure. In relation to the latter, it is worth to mention that the treatment reporting scheme changed during the study period. Before November 2011 farms were asked to report treatments only for the four pens included in the weekly report (if that was the case), indicating start and end dates for the procedures for each pen. In November 2011 it was established that the treatment report should be done at the farm level; this means that farms have to indicate the date of treatment onset in the first treated pen and the date of treatment completion in the last treated cage. In practice, the new treatment reporting design was fully adopted by all participating farms by the end of the first semester of 2012 (R. Ibarra, pers. comm.). Regardless of this change in the reporting scheme, the Official Caligidosis Surveillance and Control Program (on which the SalmonChile Monitoring Program is based) has established that whenever a treatment is carried out in a farm, it must include all active pens (i.e. pens with fish).

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## 1.6. Tables and figures

Table 1.1. Developmental stages of Caligus *rogercresseyi* under laboratory conditions at 11 °C. Gravid females were collected from the field (modified from Bravo, 2010).

| Development stage | days | Cumulative days |
|---|---|---|
| Egg string incubation | 0 | 0 |
| Copepodid (infective stage) | 9 | 9 |
| Chalimus I | 6 | 15 |
| Chalimus II | 8 | 23 |
| Chalimus III | 3 | 26 |
| Chalimus IV | 3 | 29 |
| Immature adult | 3 | 31 |
| Mature adult | 4 | 35 |

Table 1.2. Summary of currently available chemoterapeutants for control of sea lice.

| Drug | Type | Target | Via | Dose | Mechanism |
|---|---|---|---|---|---|
| Emamectin benzoate (Slice®) | Avermectin | All stages | Oral | 50 µg/kg/d, 7 consecutive days | Interfere nerve impulse (take action on chloride channels) |
| Cypermethrin (Excis®) | Synthetic pyrethroid | Attached and mobile stages | Bath | 5.0 µg/L; 1 h bath | Interfere nerve impulse (take action on sodium channels) |
| High-cis-cypermethrin (Betamax®) | Synthetic pyrethroid | (juveniles and adults) | Bath | 15 µg/L; 30 min bath | |
| Deltamethrin (Alphamax®) | Synthetic pyrethroid | | Bath | 2-3 µg/L; 40 min bath | |
| Hydrogen peroxide | Oxidizing agent | Mobile stages (removal) | Bath | 0.5 g/L, by 20 min. (not effective below 10°C) | Mechanical paralysis (bubble formation in gut and heamolymph results in detachment from the host), peroxidation of cell lipid membrane, inactivation of enzymes and DNA replication. |
| Azamethiphos (Salmosan®) | Organo-phosphate | Adult and pre-adult (not over juveniles) | Bath | 100 µg/L, by 60 min. | Neurotoxic: inhibit acetylcholinesterase (AChE) activity |
| Diflubenzuron (Lepsidon®) | Chitin synthesis inhibitors | Larval & pre-adult stages | Oral | 6 µg/kg/d, 14 consecutive days | Inhibits chitin synthesis in the copepod exoskeleton |
| Teflubenzuron (Calicide®) | Chitin synthesis inhibitors | Larval & pre-adult stages | Oral | 10 mg/kg/d bw, per 7 days (11-15°C) | |

Table 1.3. Summary of documented evidence for resistance of sea lice to antiparasitic agents in the world.

| Drug | Reported fact | Sea lice species | Region | Method | References |
|---|---|---|---|---|---|
| Dichlorvos and azamethiphos | Report of treatment failure | adult female *L. salmonis* | Scotland & Norway | Case report - case series | Jones et al., 1992; Roth et al., 1996; Fallang et al., 2004 |
| Azamethiphos | Resistance | adult female *L. salmonis* | Canada & Norway | Bimolecular rate assay | Fallang et al., 2004 |
| Pyrethroids | Report of treatment failure | *L. salmonis* | Norway, Scotland & Ireland | Case report - case series | Sevatdal et al., 2005a |
| Deltamethrin | Reduced sensitivity | *L. salmonis* | Norway | Bioassay | Sevatdal & Horsberg, 2003 |
| Deltamethin and cypermethrin | Report of treatment failure | *L. salmonis* | Ireland & Scotland | Bioassay | Sevatdal et al., 2005a |
| Deltamethrin | Reduced sensitivity | *C. rogercresseyi* | Chile | Bioassay | Helgesen et al., 2014 |
| Deltamethrin | Sensitivity differences between gender | Male and female *L. salmonis* | New Brunswick | Bioassay / survey | Whyte et al., 2014 |
| Emamectin benzoate | Reduced sensitivity | *C. rogercresseyi* | Chile | Bioassay | Bravo et al., 2008b |
| Emamectin benzoate | Reduced treatment efficacy | *L. salmonis* | Scotland | Observational (field data) | Lees et al., 2008a,b |
| Emamectin benzoate | Reduced treatment efficacy | *L. salmonis* | New Brunswick | Observational (field data) | Jones et al., 2012, 2013 |
| Emamectin benzoate | Reduced sensitivity | *L. salmonis* | New Brunswick | Bioassay | Westcott et al., 2008; Whyte et al., 2013 |
| Emamectin benzoate | Adequate treatment sensitivity / efficacy | *L. salmonis* | British Columbia | Bioassay & survey | Saksida et al., 2010, 2013 |
| Emamectin benzoate | Sensitivity differences between gender | Male and female *L. salmonis* | British Columbia | Bioassay / survey | Saksida et al., 2013 |

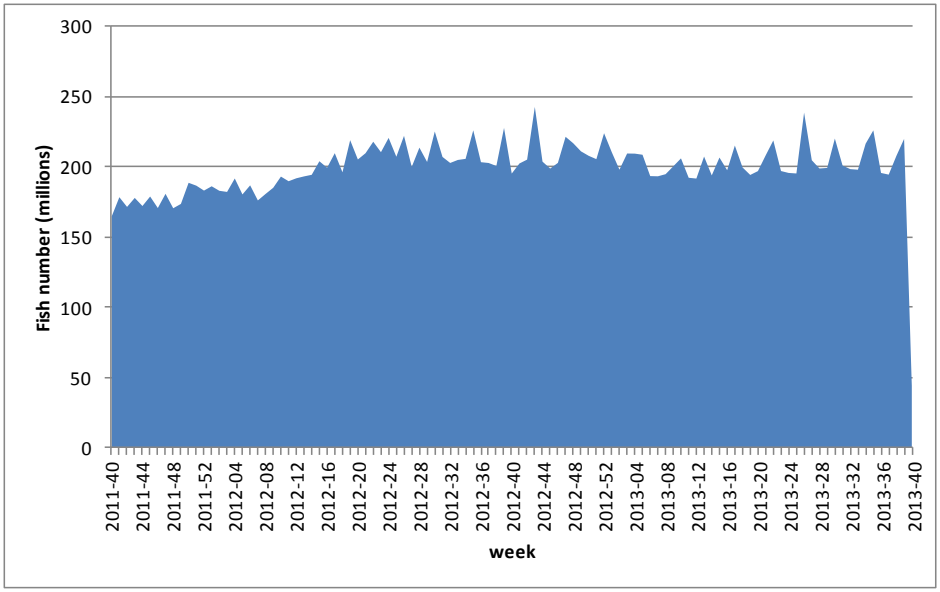


Figure 1.1. Total number of Atlantic salmon and rainbow trout fish in farms in operation in Los Lagos and Aysén regions from October 2011 to September 2013.

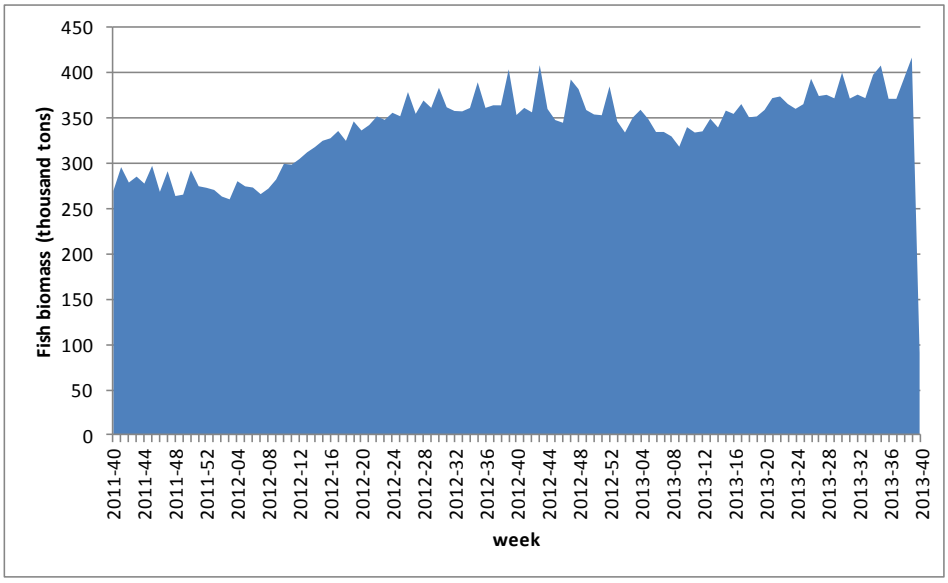


Figure 1.2. Total biomass of Atlantic salmon and rainbow trout fish in farms in operation in Los Lagos and Aysén regions from October 2011 to September 2013.

## 1.7. Appendix

### 1.7.1. Sea lice sampling protocol of the Official Caligidosis Surveillance and Control Program (excerpts) (Sernapesca, 2012)

- Sea lice sampling must be carried out by a qualified sampler, accredited for such duties by the Fisheries and Aquaculture Service (Sernapesca).
- All sea lice samples are drawn at the pen-level and consist of a 10-fish random sample. After anesthetising sampled fish a detailed lice counting is performed, considering juvenile (chalimus), mobile adults (males plus non-gravid females) and gravid females. Remaining lice in the container used for lice counting are added to the general counting.
- A pen-level general diagnostic (PLGD), which consist of sampling all active pens in the farm is carried out once during the 30 days after finishing the fish stocking. The objective of PLGD is to identify the two pens with the highest sea lice levels, which will be sampled during the rest of the production cycle. These two pens are known as "index" pens.
- For the regular sea lice monitoring four pens per farm are sampled each week for sea lice counts. Two pens are "index" pens, while the other two are randomly selected among the rest of the active pens.
- Regular sea lice monitoring starts within 30 days after finishing the fish stocking of the first stocked pen, and finishes at the harvest of the last active pen.

# CHAPTER 2

# EVALUATION OF THE PERFORMANCE OF PYRETHROIDS ON DIFFERENT LIFE STAGES OF *CALIGUS ROGERCRESSEYI* IN SOUTHERN CHILE

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(G. Arriagada designed the study, carried out the analyses, and wrote the chapter; Stryhn, H., Campistó, J.L., Rees, E.E., Sanchez, J., Ibarra, R., Medina, M., revised the manuscript; Stryhn, H., supervised the statistical analyses; and St-Hilaire, S. supervised the general work and writing)

## 2.1. Abstract

Control of sea lice in Chile is largely based on antiparasitic treatments, synthetic pyrethroids being the most used drugs. In recent years, farmers in Chile have reported decreased performance of pyrethroid-based treatments. The aim of this study was to assess the performance of two deltamethrin-based (Alphamax® and a generic product) and one cypermethrin-based (Betamax®) product on the different life stages of *C. rogercresseyi*, while controlling potential confounders. We found that both deltamethrin products and the cypermethrin product had a significant effect on the reduction of juvenile, mobile adult, and gravid female lice, compared with untreated pens; however, the effect on juvenile lice was less than on mobile stages. There was no evidence that pyrethroids performed better on certain mobile life stages, such as gravid females. When the three products were compared, no significant differences were observed in the numbers of juvenile, adult male, and non-gravid female lice after we controlled for potential confounders; however, cypermethrin exhibited a small, yet significantly greater effect on the gravid female group when compared with one of the deltamethrin-based products. We also confirmed that other factors besides the product choice, such as the pre-treatment sea lice abundance, water temperature and salinity, and time elapsed to the post-treatment sample, affect the post-treatment sea lice level as well, and therefore, they should be taken into consideration when assessing the effect of immersion treatments.

## 2.2. Introduction

Sea lice is considered one of the most economically significant parasites to salmon industries around the world (Costello, 2006, 2009). In Chile, the most important species of sea lice is *Caligus rogercresseyi*, affecting mainly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss)*, while Coho salmon (*Oncorhynchus kisutch*) appear more resistant (González et al., 2000; Bravo, 2003). Heavy infections can result in reduced growth and feed-conversion efficiency, as well as reduced marketability of fish due to skin lesions (Costello, 2009). For this reason most salmon industries, including the Chilean industry, try to maintain relatively low sea lice levels.

Sea lice control in Chile largely relies on pharmacologic treatments that target either the adult or the juvenile life stages of the parasite on the fish. Decision to treat infected fish is made at the individual farm level; however, farms within a particular farm management area (i.e. neighbourhood) are encouraged to co-ordinate their bath treatments within a few weeks of each other (Sernapesca, 2012). At present, five drugs are approved for use against *C. rogercresseyi* in Chile: emamectin benzoate (EB), deltamethrin, cypermethrin, diflubenzuron, and azamethiphos (SAG, 2013). Currently, the synthetic pyrethroids deltamethrin and cypermethrin are the most commonly used bath products for controlling *C. rogercresseyi*. These products are administered topically through immersion treatments, commonly called 'baths'. Deltamethrin is approved for mobile (adult males, non-gravid, and gravid females) stages, while cypermethrin is labelled for controlling both juvenile and mobile stages of *C. rogercresseyi* (SAG, 2013). There are two deltamethrin-based products available: Alphamax® (Pharmaq) and a generic deltamethrin (FAV), and one cypermethrin-based product, Betamax® (Novartis) (SAG, 2013). Synthetic pyrethroids are frequently combined with EB and, to a lesser extent, with diflubenzuron (R. Ibarra, pers. comm.), which targets juvenile stages. Pyrethroids interfere with nerve impulse transmission by stimulating the sodium channels in neural cells that produce spastic paralysis and subsequently death of the parasite (Corner et al., 2008; Burka et al., 2012; Torrissen et al., 2013).

As with other parasite organisms, sea lice copepods, specifically *Lepeophtheirus salmonis*, have expressed resistance patterns against synthetic pyrethroids in Norway, Scotland and Ireland, where cypermethrin and deltamethrin has been used since mid 1990s (Sevatdal and Horsberg, 2003; Sevatdal et al. 2005).

In Chile, there is no information published regarding synthetic pyrethroids efficacy or resistance of *C. rogercresseyi* to these chemotherapeutants; however, there are anecdotal reports of reduced efficacy after some pyrethroid-based treatments (R. Ibarra, pers. comm.). Greater insight about the susceptibility of particular lice development stages to specific drugs is needed, because it would ensure better selection of

products, allowing the application of targeted therapies, which may delay the development of resistance to chemotherapeutants. The aim of this study was to assess the performance of pyrethroid-based immersion treatments in Chile over the last two years and compare the three available products on the different development stages of sea lice, while controlling other important explanatory variables of the post-treatment sea lice levels.

## 2.3. Material and methods

### 2.3.1. Data

All analyses were done using data collected from the Chilean industry's sea lice monitoring program managed by the Instituto Tecnológico del Salmón (Intesal). The monitoring program records mean abundance (Rózsa et al., 2000) of total *C. rogercresseyi* by different development stages: juveniles (chalimus I, II, III and IV), mobile adults (adult males and non-gravid females), gravid females (females with egg strings), and total adults. The program also records production and environmental information from four pens per site on a weekly basis, along with delousing treatments such as the pharmaceutical product used and the date of application. Geographic coordinates of sites were also provided by Intesal. We included data from farms located in the Los Lagos and Aysén regions of Chile and information reported between October 2011 and May 2012 in our analysis.

### 2.3.2. Study design

Performance of pyrethroid-based treatments was assessed within 2 weeks after the procedure, by means of two separate studies. We evaluated three different products: Alphamax®, a generic deltamethrin, and Betamax®.

#### 2.3.2.1. Study 1: Treatment vs. negative control

In the first study, we evaluated the effect on the sea lice level of treating with a pyrethroid product versus not treating. Our study was based in sea lice treatment events, which consisted of a pair of consecutive sea

lice assessments, one before and other after a single treatment, separated by approximately one week. We only evaluated immersion treatments performed with synthetic pyrethroids. The duration of such procedures is 3 to 4 hours, depending on climatic conditions and other factors. To be included in this study, each treatment event also had to have at least one matched consecutive pair of sea lice assessments with no delousing treatments at all (i.e. an untreated event), selected from a different pen within the same farm and within ± 2 weeks from the treatment date. We included up to three untreated events per treatment. The design was a matched cohort study, with the sea lice mean abundance at the post-treatment sample as the outcome. The exposure variable was the pyrethroid product choice, coded as CM, DM1, and DM2, plus the untreated option. Treatments were done by the producers, based on their own criteria, and following the manufacturer directions. Although the information is not complete, we know that the majority of treatment events included in this study were performed with the skirt method. Weekly sea lice assessments were performed following the protocols described in the Official Caligidosis Surveillance and Control Program (Sernapesca, 2012). Separate analyses were done for each juvenile (chalimus), mobile adults (adult males and non-gravid females), and gravid female lice.

#### 2.3.2.2. Study 2: Comparing pyrethroid-based products

The second study compared the performance of the three pyrethroid products against each other. For descriptive purposes we estimated the overall efficacy[1] of pyrethroids by sea lice development stages. In this study, we only included treatment events in which the pre-treatment sea lice sample was taken 1 to 3 days before the treatment procedure, and the post-treatment sample was taken 3 to 14 days after the treatment. The design was a cohort study, with the mean sea lice abundance at the post-treatment sample as the outcome of interest. The exposure variable was the product used in the treatment (i.e. CM, DM1, and DM2). As with the first study, separate analyses were done for each of the three sea lice life stages.

[1] Efficacy = (pre-treatment level – post-treatment level) / pre-treatment level

### 2.3.3. Controlling for confounders

Because sea lice infections can also be influenced by biological, environmental, and production factors, we included other variables in the analysis to control for confounding. In all models, we included the log of the mean sea lice level before the treatment (as determined by the pre-treatment sample) as an explanatory variable to control for pre-treatment exposure. We also included the abundance of immature lice at the pre-treatment sample as an explanatory variable to account for the natural development of the parasite over time. With the same purpose, the time elapsed between the treatment procedure and the post-treatment sample was tested in the model. The salmonid species (i.e. rainbow trout or Atlantic salmon) was included as a dichotomous variable, because differences in susceptibility to sea lice have been reported (González et al., 2000; Bravo, 2003). Among environmental factors, water temperature (° C) and salinity (ppm) were assessed in the analysis, because these parameters can have an effect on the maturation rate and fecundity of *C. rogercresseyi* (González and Carvajal, 2003; Bravo, 2010). Given that it has been reported *C. rogercresseyi* may be transmitted up to 30 km through the sea (Kristoffersen et al., 2013), the number of active sites within a seaway distances of 30 km was included for each site in the model as a proxy variable for the infestation pressure. Seaway distances were calculated using the *gdistance* package in R v.3.0.1 (*www.r-project.org*). We assessed and controlled for effects of production factors such as mean fish weight (kg), fish biomass (tons), stocking density (fish per $m^3$), and the total number of fish (in thousands) given their previously described associations to *C. rogercresseyi* abundance (Zagmutt-Vergara et al. 2005; Yatabe et al. 2011; Kristoffersen et al., 2013). For these variables, we took the value at the pre-treatment sample. In addition, previous delousing treatments during four weeks before the treatment in evaluation in the same pen were included as a 5-level categorical variable with the following categories: no treatment, unknown (if time series was incomplete), diflubenzuron treatment, emamectin benzoate (EB) treatment, and pyrethroid treatment. As sea lice levels might show large-scale spatial variation, we also included the region with 2 categories. As data presented a hierarchical structure, we tested company, farm, and pen random effects to control for the clustering of sea lice. In the first study where we matched the treatment events we also tested a random effect for the

matching group at the bottom of the hierarchy.

### 2.3.4. Statistical models

Data were analyzed using linear mixed models. The post-treatment sea lice abundance (outcome of interest) was log transformed to improve the fit of the linear models. Pre-treatment sea lice mean abundance was also log transformed and included as an explanatory variable. Potential confounders, other explanatory variables, including random effects were tested in models when appropriate.

### 2.3.5. Model building and model validation

Models were built using a stepwise backward elimination process starting with a maximum model containing all relevant predictors. The least significant variables (higher Wald p-value) were removed from the model one at a time until all remaining variables were significant (Wald-test $p \leq 0.05$), unless substantial changes (> 20%) in coefficient of other variables were noticed. The exposure variable (pyrethroid product) was retained in all models regardless of its p-value. The same criterion was used for the pre-treatment sea lice levels that were included in all models as predictors. Models were fitted using restricted maximum likelihood (REML) estimation. Variance estimates for random effects were tested with likelihood ratio tests based on REML. Random effects were retained if the variance estimate was different from zero. Random effects for lower levels (i.e. matching group and pen) were included in sensitivity analyses but were left out from the final models if they had a negligible impact on coefficients. Collinearity between explanatory variables was assessed during the stepwise backward elimination process. When two highly ($|\rho|>0.7$) correlated predictors where observed, one of them was retained in the model based on biological, practical or statistical criteria (in that order). Linearity between continuous predictors and the outcome was checked by running mean and lowess smoothed-lines between the standardized residuals and each continuous predictor retained in the final model. Two-way interactions were tested between pyrethroid product and pre-treatment sea lice abundance, species, mean fish weight, water temperature, and water salinity. Homoscedasticity and normality of residuals for our final models

were checked for both random effects and error term. Homoscedasticity was examined by plotting standardized residuals vs. fitted values. Normality of residuals was evaluated by a Q-Q plot using standardized residuals. Unusual observations (absolute value for standardized residuals > 3) were identified and excluded in a sensitivity analysis for the final model in order to assess unduly impact over the coefficients. Pairwise comparisons between the three products in evaluation were carried out only for the second study when the overall *p*-value was significant, using the Bonferroni method for adjusting the level of significance. All statistical analyses were performed with Stata version 13.

## 2.4. Results

### 2.4.1. Study 1: Treatment vs. negative control

A total of 76 treatment events and 110 no-treatment events met the selection criteria. Among treatment events, 37 were performed with CM, 16 with DM1, and 23 with DM2. Data originated from 161 pens, 55 sites, and 14 companies located in Los Lagos (n=42) and Aysén (n=144) regions between October 2011 and May 2012. In most cases, each treatment event was originated from a single pen; whereas in 7 instances, a single pen provided more than one treatment event (maximum 3 treatment events per pen).

When no treatment was given, the mean sea lice abundance between sample periods on average either remained similar (for juvenile sea lice stages) or increased (for mobile stages). In contrast, when a pyrethroid treatment was administered, on average the mean abundance sea lice level decreased for all lice stages; however, the decrease was most evident for the adult lice stages (Figure 2.1). Environmental and production characteristics recorded during treatments are summarized in Table 2.1.

Pens treated with one of the three pyrethroid products had significantly lower mean juvenile, mobile adult, and gravid female sea lice abundance after treatment compared to the untreated pens (Table 2.2). In general, the effect of treating seemed to be less strong for juvenile than mobile sea lice in each of the three products evaluated. For instance, when treatments were performed with cypermethrin (CM), the

post-treatment level was, on average, 65% ($1-e^{-1.062}$) lower for juvenile lice, 88% lower for mobile adults, and 90% lower for mobile lice, compared to untreated pens. Further, in the particular case of mobile adults the effect of treatments performed with deltamethrin (DM1 or DM2) was dependent on the pre-treatment sea lice level, being greater when sea lice abundance before the treatment was higher.

Other explanatory variables showed a significant association with the outcome. Sea lice level before the treatment and mean fish weight were positively associated with the sea lice level after treatment for juvenile, mobile adult, and gravid female lice. Water temperature exhibited a negative association with the post-treatment sea lice level, only for the juvenile group. Random effects for company and site were not significant in all life stages, suggesting there was no substantial clustering of sea lice at these levels. Random effects for matching group and pen were dropped since the number of treatment events within these groups was fairly small.

### 2.4.2. Study 2: Comparing pyrethroid-based products

For this study, we selected 181 treatment events, of which 106 were done with CM, 32 with DM1, and 43 with DM2. These treatments were performed in 132 pens, 73 sites, and 12 companies between October 2011 and May 2012 in the Los Lagos (n=61) and Aysén (n=120) regions in southern Chile. Ninety-seven pens provided one treatment event, 23 provided 2 treatments, 10 provided 3 treatments, and only 2 provided 4 treatments. The efficacy of pyrethroid treatments on juvenile, mobile adults and gravid female lice were 0.24 (sd=1.26), 0.64 (sd=1.01), and 0.75 (sd=0.81), respectively.

Similar to the subset of data on sea lice treatments used in our first study, the abundance of all sea lice stages decreased after treatment with all three products evaluated. Table 2.1 shows descriptive statistics on the environmental and production conditions during the treatment procedures.

Overall *p*-values for the pyrethroid-based products were significant only for gravid female lice (see Table 2.3). Pairwise comparisons showed that CM was associated with significant lower levels of lice than the DM2 treated fish. The abundance of gravid female level following a cypermethrin treatment was, on average, 36% ($1-e^{-0.439}$) lower than the level in fish treated with DM2 (Table 2.3).

Other explanatory variables significantly associated with sea lice abundance after treatments were pre-treatment sea lice levels for the life stage being evaluated and for previous stages in the life cycle. For example, both the juvenile lice and the gravid female lice levels before the treatment were positively associated with the gravid female lice level after treatment. Water temperature was negatively associated with juvenile lice after treatment, while water salinity exhibited a positive association with gravid female post-treatment abundance. Mean fish weight and time elapsed from the treatment procedure to the post-treatment sample were positively associated only with mobile lice. The effect of farming activity in a 30 km radius from the site in evaluation on the post-treatment sea lice level was positive and only significant in the case of juvenile and mobile adult (adult male and non-gravid female) lice. Moreover, when fish were treated with EB within one month prior the pyrethroid treatment in evaluation, the mobile adult lice level was even lower. No significant differences were observed with previous treatments with other antiparasitic drugs. Significant differences in the sea lice levels across regions were observed only for adult life stages, while differences across sites were exhibited in all life stages. Furthermore, in the case of juvenile lice, there were significant differences across companies as well. Random effects for pen were dropped as the variance estimate was zero for all life stages.

## 2.5. Discussion

We evaluated the performance of pyrethroid-based treatments for controlling *C. rogercresseyi* in Atlantic salmon and rainbow trout farms in southern Chile. The first major finding of this study was that each of the three products evaluated was associated with a significantly lower juvenile, mobile adult, and gravid female lice level after treatment, when compared with an untreated pen; however, the three products

appeared to be less effective at reducing the number of juvenile lice compared to mobile stages.

Better results of pyrethroids on mobile lice could be due to the fact that juvenile lice, especially chalimus I and II stages, may be protected from bath treatments by the mucus covering on the fish. Scientific literature regarding the effect of pyrethroids on *C. rogercresseyi* is limited; however, there are several studies that have examined their effect on *Lepeophteirus salmonis*. For example, in agreement with our findings, Hart et al. (1997), Roth (2000) and Stone et al. (1999, 2000) reported a higher reduction in mobile *L. salmonis* stages compared to juvenile lice after pyrethroid administration. Whether this effect is sufficient to warrant recommending the use of pyrethroids to control juvenile *C. rogercresseyi* is a subjective matter. In any case, we know from this study that some juvenile lice will remain on the fish after treatment; therefore, the life cycle will continue developing in the pen. On the other hand, our results suggest that, on average, pyrethroids have a similar effect on different *C. rogercresseyi* mobile life stages. However, since the effect of deltamethrin on adult male and non-gravid female lice seemed to be dependent on the pre-treatment sea lice abundance, the performance of this drug on mobile stages may vary (Table 2.2). This finding disagreed with a study by Sevatdal et al. (2005) who reported important differences in the effect of pyrethroids on *L. salmonis* mobile stages, the adult female group being less sensitive than pre-adult II lice. Similarly, Hammell et al. (2011) reported that deltamethrin was more effective on pre-adult and adult males than on adult female *L. salmonis*. A possible explanation for this discrepancy is the sensitivity of mobile sea lice to pyrethroids appears to be size-dependent (Sevatdal & Horsberg, 2003), being larger lice less sensitive than smaller lice. In the case of *L. salmonis*, the female louse is twice the size of the male (Johnson & Albright, 1991), whereas the female *C. rogercresseyi* is practically the same size as the male (Boxshall & Bravo, 2000).

The second main finding of our study was that, after adjusting for important explanatory variables, the three products under evaluation seemed to be equally effective on both juvenile and mobile adult (adult males and non-gravid females) *C. rogercresseyi*. This was unexpected because in Chile cypermethrin is

labelled for all sea lice life stages, while deltamethrin is approved only for the adult group (SAG, 2013). Similar labelling regulates the use of pyrethroids against *L. salmonis* in Europe (IMB, 2013; VMD, 2013). In contrast, cypermethrin exhibited a significantly greater effect on the gravid female group than DM2. Differences on the effect of cypermethrin and deltamethrin on a particular sea lice life stage have been reported by Sevatdal et al. (2005) who revealed that *L. salmonis* pre-adults II were less sensitive to cypermethrin, compared to deltamethrin. Whether the difference observed between products on the gravid female group has practical implications for sea lice control is unknown and should be addressed in future research; however, since the gravid female group are responsible for sea lice larvae production on the farms, targeting these might reduce the amount of copepodid in the water two or three weeks after the treatment procedure. Such a difference might be explained by a specific effect of cypermethrin on gravid female lice or by reduction of sensitivity of *C. rogercresseyi* to deltamethrin given this drug has been used for a longer period of time than cypermethrin in Chile.

Effective control of sea lice on farms is based on the strategic use of pharmaceutical options against specific sea lice life stages. For example, Treasurer and Grant (1997) and Jackson (1998) suggest that pharmacological treatments should be targeted to juvenile lice, rather than other stages. On the other hand, the Sea Lice Resistance to Chemotherapeutants (SEARCH) Project (2006) concluded that gravid females should be targeted using specific delousing agents in order to reduce them to an absolute minimum during winter time. In any case, knowledge about the sensitivity of the parasite to a given drug is a crucial element in deciding which product to use (Grant, 2002). In this sense, our study has provided important information regarding effects of pyrethroids on specific *C. rogercresseyi* life stages. From a practical point of view, our study suggests that if pyrethroids are used as a single treatment for controlling *C. rogercresseyi*, the life cycle will not be completely interrupted because of the survival of juvenile lice that will develop into adults in one or two weeks, depending on the water temperature.

Another important outcome of our study was that we confirmed that other factors besides the product choice did influence post-treatment sea lice levels. If these are not taken into consideration, they could bias results on treatment evaluation. Among biological factors evaluated, we found that the level of sea lice at the pre-treatment sample for both the life stage evaluated and for previous life stages (i.e. juvenile level predicts mobile adult level) were positively associated with the sea lice level after the treatment. These two variables, along with the time elapsed from the treatment procedure to the post-treatment sample, accounted for the natural progression of the sea lice life cycle between the two samples. Further, since time elapsed was not significant in the juvenile lice model, we can infer that new settlement of sea lice, coming from the same farm or elsewhere, did not affect the post treatment counts in our study. Given the warmest scenario (15°C) chalimus III might be seen after 12 days (Bravo, 2010), and inclusion of younger chalimi (I and II) in the juvenile sea lice count is unlikely due to its small size.

Water temperature showed a negative effect on juvenile lice abundance like other observational studies conducted on juvenile stages on *C. rogercresseyi* (Kristoffersen et al., 2013). A possible explanation for this finding is that after a treatment, remaining juvenile lice develop into older stages faster with higher temperatures and the time period from the treatment procedure to the post-treatment sampling is too short for observing new juvenile lice infections from external sources (could also be from within the farm). Water salinity was positively associated with gravid female lice abundance which is consistent with conclusions drawn by Bravo et al. (2008) who found that salinity was directly associated with survival of adult *C. rogercresseyi*.

Other factors affecting the post-treatment sea lice level were the fish mean weight (Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013), and the number of active farms within a 30 km radius. The latter was a measure of the infestation pressure (new juvenile stages), which is consistent with the conclusions of Kristoffersen et al. (2013). Previous delousing treatments should also be considered when assessing treatments. In our case, we found that when an emamectin benzoate was performed

during the month before the pyrethroid treatment, the level of mobile adults was lower. Finally, we also found significant differences in the sea lice level by region, company, and site, which agreed with studies by Zagmutt-Vergara et al. (2005), Hamilton-West et al. (2011) and Yatabe et al. (2011).

All reported treatments that met the selection criteria (see section 2.3.2) were included in the study; however due to the selection criteria we did not randomly select from all treatments performed in the industry database and this may have introduced selection bias. However, it is unlikely that performance of selected treatments is significantly different from the rest of treatments reported in the Intesal's system between October 2011 and May 2012; therefore we believe selection bias was negligible. Measurement error of the sea lice counting is likely to be present, particularly in the case of juvenile lice, because of their small size; nevertheless, it is unlikely to be differential among the three products in evaluation; therefore, if this bias occurred it would have biased towards the null. Confounding was controlled through matching and analytic control. However, the information regarding the bath method (skirt, tarpaulin, or well boat) was not available and therefore could not be controlled in our analysis. In any case, there is no reason to suggest that product choice is associated with a particular bath method (R. Ibarra, pers. comm.) so we have no reason to believe omission of this predictor would have changed our conclusions.

## 2.6. Conclusions

Knowledge about the effect of delousing drugs over specific development stages of sea lice is a key factor for a successful control strategy. The main contribution of this study is that it presents the first investigation on the effect of pyrethroids on different life stages of *C. rogercresseyi*. Our study suggests that during 2011 and 2012 approved pyrethroids in Chile were more effective on adult than juvenile lice. Unlike what has been reported for *L. salmonis*, pyrethroids seemed to have similar effects on both gravid female lice and the rest of mobile life stages of *C. rogercresseyi*. When comparing the three products on specific life stages, no significant differences in performance were observed on juvenile, adult male and non-gravid female lice; however, CM exhibited a significantly greater performance on the gravid female

group when compared with DM2.

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## 2.8. Tables and figures

Table 2.1. Descriptive statistics of the production and environmental variables tested in both the treatment vs. negative control study and in the comparison between pyrethroid-based products study.

| Variables | Study 1[a] | | | Study 2[b] | | |
|---|---|---|---|---|---|---|
| | Mean | 90% range | n | Mean | 90% range | n |
| Water temperature (°C) at the pre-treatment sample | 11.8 | 9.9 – 13.8 | 186 | 11.9 | 9.9 – 13.8 | 181 |
| Water salinity (ppm) at the pre-treatment sample | 31 | 26 – 33 | 186 | 31 | 27 – 33 | 181 |
| Fish weight (kg) | | | | | | |
| Atlantic salmon | 2.24 | 0.24 – 4.73 | 158 | 2.04 | 0.28 – 4.87 | 146 |
| Rainbow trout | 1.94 | 0.48 – 3.00 | 28 | 2.07 | 0.32 – 3.09 | 35 |
| Fish biomass (tons) at the pre-treatment sample | 92.9 | 13.1 – 222.0 | 186 | 87.6 | 14.4 – 184.4 | 181 |
| Stocking density (kg/$m^3$) at the pre-treatment sample | 7.0 | 0.9 – 14.0 | 186 | 6.4 | 1.11 – 13.2 | 181 |
| Fish number (thousands) at the pre-treatment sample | 44.2 | 26.3 – 61.7 | 186 | 44.7 | 30.5 – 58.0 | 181 |
| Time between samples (days) | 7.0 | 6 – 8 | 186 | 6.8 | 5 – 8 | 181 |
| Timing of sea lice sampling regarding the treatment procedure | | | | | | |
| Days before | 3.7 | 1 – 7 | 76 | 1.6 | 1 – 3 | 181 |
| Days after | 3.4 | 1 – 6 | 76 | 5.2 | 3 – 7 | 181 |
| Number of neighbouring farms in a radius of 30 km | 28.9 | 6 – 62 | 180 | 35.7 | 8 – 71 | 180 |

[a] Treatment (n=76) vs. negative control (n=110) study.
[b] Comparison between pyrethroid-based products study (n=181).

Table 2.2. Coefficient estimates, standard errors and *p*-values for explanatory variables in the final model for the treatment vs. control analysis.

| Variable name | Juveniles | | | Mobile adults | | | Gravid females | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coefficient estimate | Standard error | *p*-value | Coefficient estimate | Standard error | *p*-value | Coefficient estimate | Standard error | *p*-value |
| **Fixed effects** | | | | | | | | | |
| Intercept | 1.696 | 0.721 | | 0.082 | 0.140 | | 0.137 | 0.139 | |
| Pyrethroid-based product (no-treatment as reference) | | | **<0.001** | | | **<0.001** | | | **<0.001** |
| CM | -1.062 | 0.178 | **<0.001** | -2.088 | 0.191 | **<0.001** | -2.328 | 0.189 | **<0.001** |
| DM1 | -0.957 | 0.237 | **<0.001** | -1.807[a] | 0.258 | **<0.001** | -1.646 | 0.256 | **<0.001** |
| DM2 | -0.560 | 0.214 | **0.009** | -1.524[a] | 0.226 | **<0.001** | -1.492 | 0.227 | **<0.001** |
| Log of juvenile mean abundance at pre-treatment sample | 0.475 | 0.079 | **<0.001** | 0.250 | 0.082 | **0.002** | | | |
| Log of mobile adult mean abundance at pre-treatment sample | | | | 0.580 | 0.084 | **<0.001** | | | |
| Log of gravid female mean abundance at pre-treatment sample | 0.155 | 0.069 | **0.025** | | | | 0.660 | 0.068 | **<0.001** |
| Water temperature at the pre-treatment sample (° C) | -0.121 | 0.060 | **0.044** | | | | | | |
| Fish mean weight in kg, centered | | | | 0.115 | 0.049 | **0.019** | 0.118 | 0.049 | **0.017** |
| **Random effects / Variances** | | | | | | | | | |
| Company | 0.033 | 0.064 | 0.275 | | | | | | |
| Site | 0.114 | 0.093 | 0.067 | 0.034 | 0.056 | 0.248 | 0.036 | 0.061 | 0.259 |
| Error | 0.649 | 0.088 | | 0.802 | 0.097 | | 0.804 | 0.099 | |

[a] Due to an interaction between product and pre-treatment mobile adult abundance, the coefficient estimates for 'DM2' and 'DM1' are average values based on linear combinations of coefficients using the product specific mean value of the log mobile adult abundance at pre-treatment sample; 90% ranges of the combined coefficient values are -1.134 to -1.932 (DM2), and -1.118 to -2.496 (DM1).

Table 2.3. Coefficient estimates, standard errors and *p*-values for explanatory variables in the final model for the comparison between products analysis.

| Variable name | Juveniles | | | Mobile adults | | | Gravid females | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coefficient estimate | Standard error | *p*-value | Coefficient estimate | Standard error | *p*-value | Coefficient estimate | Standard error | *p*-value |
| **Fixed effects** | | | | | | | | | |
| Intercept | 1.500 | 0.811 | | -2.097 | 0.406 | | -4.755 | 1.258 | **<0.001** |
| Pyrethroid-based product (DM2 as reference) | | | 0.419 | | | 0.629 | | | **0.013** |
| CM | -0.210 | 0.212 | 0.322 | -0.104 | 0.184 | 0.574 | -0.439 | 0.172 | **0.010** |
| DM1 | 0.068 | 0.249 | 0.784 | 0.104 | 0.242 | 0.666 | 0.011 | 0.231 | 0.963 |
| Log of juvenile mean abundance at pre-treatment sample | 0.251 | 0.088 | **0.004** | 0.411 | 0.082 | **<0.001** | 0.206 | 0.080 | **0.010** |
| Mobile adult mean abundance at pre-treatment sample | 0.041 | 0.020 | **0.039** | | | | | | |
| Log of gravid female mean abundance at pre-treatment sample | | | | | | | 0.141 | 0.085 | 0.098 |
| Time elapsed from treatment procedure to post-treatment sample in days | | | | 0.115 | 0.050 | **0.022** | 0.147 | 0.048 | **0.002** |
| Water temperature at pre-treatment sample | -0.188 | 0.060 | **0.001** | | | | | | |
| Water salinity at pre-treatment sample | | | | | | | 0.089 | 0.040 | **0.027** |
| Region (Aysén as reference) | -0.638 | 0.341 | 0.061 | -1.020 | 0.291 | **<0.001** | -0.690 | 0.183 | **<0.001** |
| Number of farms within a radius of 30 km | 0.017 | 0.008 | **0.023** | 0.015 | 0.007 | **0.032** | | | |
| Fish mean weight in kg, centered | | | | 0.132 | 0.066 | **0.044** | 0.234 | 0.057 | **<0.001** |
| Previous treatments within the previous month | | | | | | 0.059 | | | |
| no treatment (reference) | | | | | | | | | |
| no-information | | | | 0.238 | 0.168 | 0.157 | | | |
| diflubenzuron | | | | -0.096 | 0.510 | 0.851 | | | |
| EB | | | | -0.740 | 0.360 | **0.039** | | | |
| pyrethroids (previous 2 weeks) | | | | 0.546 | 0.346 | 0.114 | | | |
| **Random effects / Variances** | | | | | | | | | |
| Company | 0.266 | 0.206 | **0.004** | | | | | | |
| Site | 0.373 | 0.118 | **<0.001** | 0.455 | 0.132 | **<0.001** | 0.298 | 0.088 | **<0.001** |
| Error | 0.394 | 0.055 | | 0.384 | 0.057 | | 0.400 | 0.055 | |

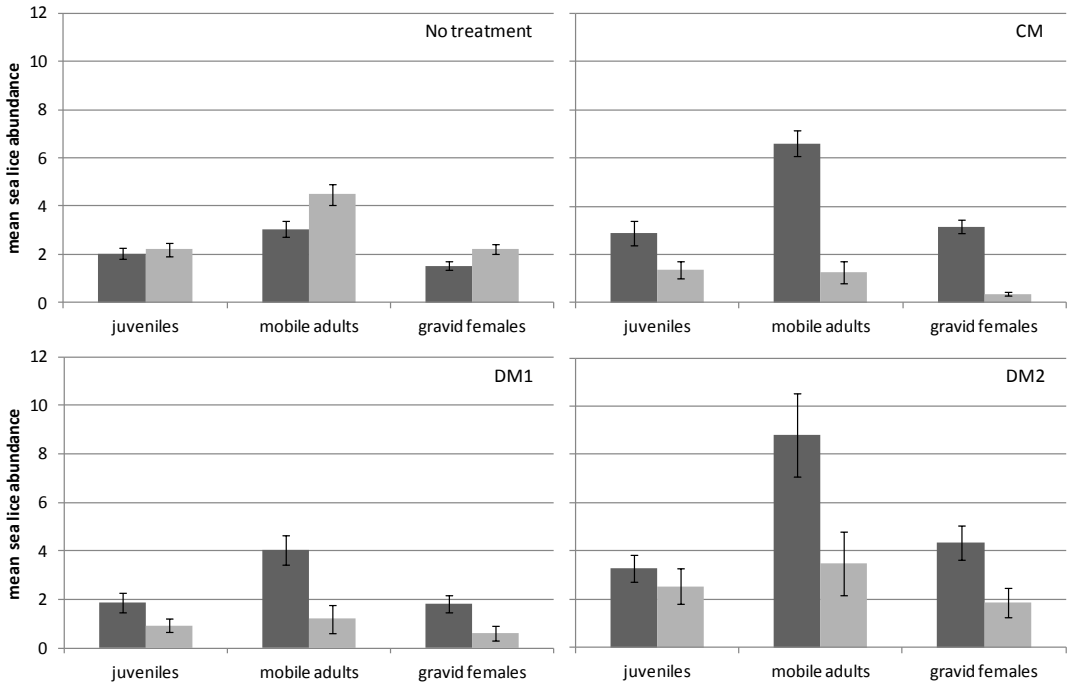


Figure 2.1.*Caligus rogercresseyi* juvenile, mobile adult and gravid female mean abundances with simple standard error bars in two consecutive samples separated by approximately one week with no antiparasitic treatment, or with a single treatment procedure with either cypermethrin (CM), deltamethrin from laboratory 1 (DM1) or deltamethrin from laboratory 2 (DM2).

## 2.9. Appendix

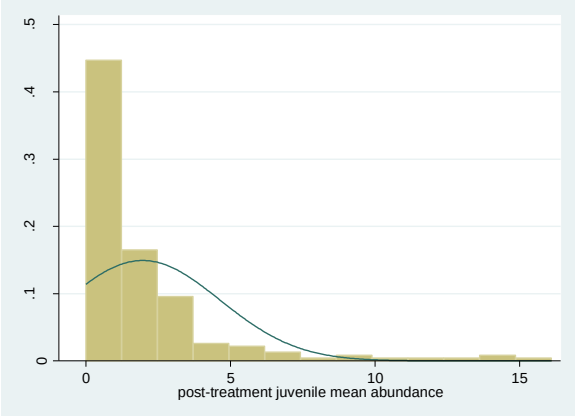


Figure 2.2a. Histogram for post-treatment juvenile mean abundance (raw data).

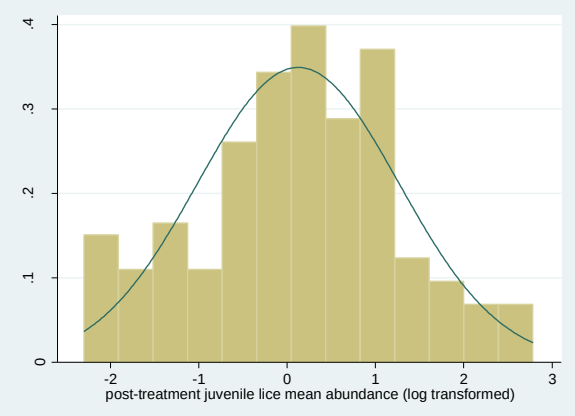


Figure 2.2b. Histogram for post-treatment juvenile mean abundance (log transformed data).

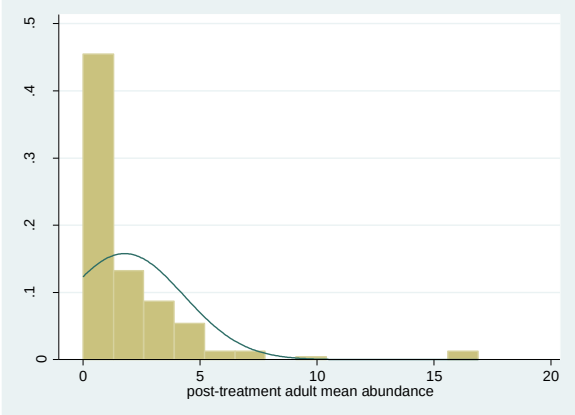


Figure 2.3a. Histogram for post-treatment mobile adult mean abundance (raw data).

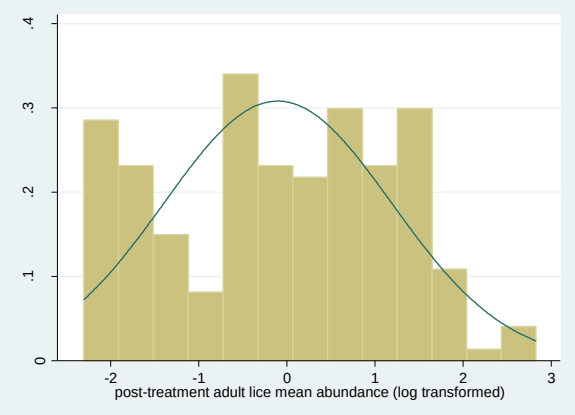


Figure 2.3b. Histogram for post-treatment mobile adult mean abundance (log transformed data).

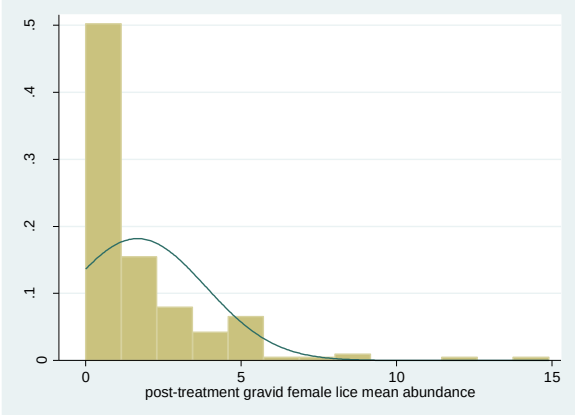


Figure 2.4a. Histogram for post-treatment gravid female mean abundance (raw data).

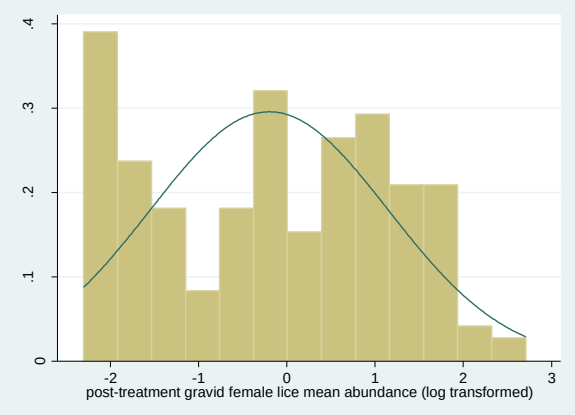


Figure 2.4b. Histogram for post-treatment gravid female mean abundance (log transformed data).

# CHAPTER 3

## A MULTIVARIABLE ASSESSMENT OF THE SPATIO-TEMPORAL DISTRIBUTION OF PYRETHROIDS PERFORMANCE ON THE SEA LICE *CALIGUS ROGERCRESSEYI* IN CHILE

## 3.1. Abstract

Sea lice is the most economically important ectoparasite affecting farmed salmon worldwide. Pharmacologic treatments are the main strategy for controlling sea lice, synthetic pyrethroids being one of the first choices in many regions such as Chile. Researches have reported pyrethroid treatment failures since 2008, and resistance of *Caligus rogercresseyi* to deltamethrin in 2012. However, there is no information regarding the geographic extent of the problem. In this study, we explored the spatial and temporal variation of *C. rogercresseyi*'s response to pyrethroid treatments in Chile between 2012 and 2103 and examined factors related to this variability. We modeled the adult lice level one week after treatment with a linear mixed-effects regression, controlling for several management and environmental variables, including pre-treatment sea lice levels. We used the model to predict farm level effects and residuals at the treatment level and subjected these to a purely spatial and to a spatio-temporal cluster analysis, respectively. The Moran's *I* coefficient suggests farms closer to one another have more similar farm effects, while the scan spatial statistic suggests there were two areas, one located in northern Los Lagos region, and the other in central Aysén region, where the post-treatment adult lice level attributed to the farm effect was significantly higher than in the rest of the study area. These spatial clusters remained even once we adjusted for environmental and management predictors, suggesting unknown factors were causing the clustering in these areas. One potential factor that may explain the clustering is that sea lice populations in these areas are more tolerant to pyrethroids. Further investigation should be carried out to confirm or rule out this situation.

## 3.2. Introduction

The copepods *Lepeophteirus salmonis* and *Caligus rogercresseyi*, commonly called sea lice, are considered the most important ectoparasites that affect farmed salmonids around the world (Costello, 2006; Burka et al., 2012). Although rarely life threatening, when sea lice are numerous on fish they can produce extensive skin damage, osmotic stress, and increase susceptibility to secondary infections

(González et al., 2000; Johnson et al., 2004; Costello, 2006). Cost increases are related to reduction of growth, reduced feed-conversion efficacy of fish, delousing treatments, and reduced marketability of the final product (Costello, 2009).

Pharmacologic treatments are the most wide-spread strategy used against sea lice infestations in farmed salmonids (Torrissen et al., 2013). The most-used drugs in the last decade are emamectin benzoate (EB), the synthetic pyrethroids deltamethrin and cypermethrin, and the organophosphate azamethiphos (Burridge et al., 2010). However, treatment failures have been reported in most of the larger salmon-producing regions of the world for many drugs (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005; Bravo et al., 2008b; Lees et al., 2008a,b; Jones, 2012, 2013; Bravo et al., 2013).

Subsequent research has revealed that one of the causes for treatment failures is the decrease of sensitivity of some sea lice populations towards several of these chemotherapeutants. So far, populations of *L. salmonis* have demonstrated to be less sensitive to Emamectin benzoate (Westcott et al., 2008; Whyte et al., 2013), deltamethrin (Sevatdal et al., 2005; Whyte et al. 2014), cypermethrin (Sevatdal et al., 2005), and azamethiphos (Fallang et al. 2004). In Chile, low sensitivity or resistance of the sea lice *C. rogercresseyi* has been reported towards EB (Bravo et al., 2008b; Bravo et al., 2010), and more recently to deltamethrin (Helgesen et al., 2014).

Evaluation of treatment efficacy is a core activity in sensitivity monitoring (SEARCH project, 2006; Jackson et al., 2011). Studies on EB efficacy in Scotland and New Brunswick (NB), Canada, have shown differences across geographical regions (Lees et al., 2008a,b; Jones et al., 2012). Some of these studies have shown that treatment becomes less effective as fish age (Lees et al., 2008a; Jones et al., 2012; Saksida et al., 2013), which could mean that tolerance increases over the production cycle.

Other causes of treatment failure are related to problems with drug administration and re-infestation shortly after the procedure. Some common problems associated with drug administration for immersion treatments include insufficient reduction of the pen volume during treatment resulting in dilution of the product, improper use of tarpaulin, and premature evaluation of treatment (i.e. post-treatment sample is taken too early). In the case of oral treatments, a common pitfall is errors in dosage rate (SEARCH project, 2006).

Re-infestation occurs when new sea lice colonize fish shortly after a treatment, so the sea lice level at the post-treatment sample is higher than expected. Because drugs used in immersion treatments do not confer a long lasting effect, these types of treatments are more prone to be negatively affected by re-infestation than oral treatments, especially in areas with intense salmon farming activity (SEARCH project, 2006). Recently, a study by Kristoffersen et al. (2013) found the infectious pressure from neighbouring farms to be significant source of lice for farms.

The external infectious pressure has recently included in sea lice epidemiologic research, and it has been expressed in different ways. Jansen et al. (2012), for example, investigated the importance of local biomass density (LBD) of farmed salmonids on mobile *L. salmonis* abundances in Norway. LBD incorporated the fish biomass in 40 km seaway distance. Neighbouring farm contributions were weighted with a Gaussian kernel densitiy based on distance between farms. In a study aimed to investigate different sources of sea lice in Chile, Kristoffersen et al. (2013) expressed the external infectious pressure as the product between the mean gravid female lice and the number of fish at neighbouring farms, using weights similar to what was used by Jansen et al. (2012). A similar approach was used by Rees et al. (2015) when investigating spatial patterns of sea lice infections in western Canada. In a much more complex model formulation, Aldrin et al. (2013) quantified the contribution of each neighbouring farm to the sea lice level by considering a density function for the seaway distance between farms, the number of fish and several lagged previous sea lice abundances at the neighbouring farms. The between-farms distance

function was not defined *a priori*, but was modeled by 2 parameters that allowed different functional shapes. The estimated distance function closely followed an exponential shape.

Treatment success generally is evaluated by comparing the sea lice levels before and after treatment. When information about the possible drivers of treatment outcome is available, multivariable techniques may help to quantify the contribution of each of predictor on treatment result. Multivariable approaches have been used to evaluate the performance of treatments with Emamectin benzoate in Scotland (Lees et al., 2008a,b), and New Brunswick, East Canada (Jones et al., 2012, 2013), and for treatments with pyrethroids in Chile (Chapter 2).

The evaluation of spatial clustering of treatment performance is important because it may inform about the nature of factors that drive a spatial dependence. The combination of multivariable techniques and spatial statistics may help to generate hypotheses about factors affecting treatment performance and their distribution in space. The objective of this study was to explore the spatial and spatio-temporal variation of *C. rogercresseyi* response to pyrethroid treatments and to examine factors related to this variability.

## 3.3. Methods

We evaluated the adult lice abundance one week after a pyrethroid bath treatment, while controlling for other factors that affect sea lice abundance such as pre-treatment levels, environmental and management variables. We subsequently evaluated whether this adjusted treatment performance was clustered in space and time within the Chilean salmon industry.

### 3.3.1. Data

All data for the analyses were obtained from the Chilean salmon industry's Sea Lice Monitoring Program, administered by the Instituto Tecnológico del Salmon (Intesal), which gathers health and production data from more than 90% of the salmon farms currently in operation. Each participating farm reports *C.*

*rogercresseyi* counts of juvenile (chalimus I to IV), mobile adults (male and non-gravid females) and gravid female lice, from a 10-fish sample each drawn from four pens on a weekly basis (40 fish in total). Sampling followed the protocols of the Official Caligidosis Surveillance and Control Program (Sernapesca, 2012). Participating farms also report delousing treatments, including the drug used, and start and end dates of the procedure at the farm level. In addition, fish production and environmental information, such as mean fish weight, total number of fish, fish biomass, water salinity and water temperature, are also reported on a weekly basis. Our study was restricted to data collected from January 2012 to September 2013, due to a change in the treatment reporting scheme before that period.

#### 3.3.1.1. Selection of treatments

We selected delousing treatments performed only with synthetic pyrethroids, either deltamethrin or cypermethrin, from Atlantic salmon or rainbow trout production cycles whose onset was dated within our study period. We discarded single treatment procedures that were reported over a period greater than 16 days, because longer treatments are not common in the Chilean industry and the effect of the treatment as measured by pre- and post-treatment sea lice counts could not be accurately determined with the data provided. According to the legislation in force during 2012 and 2013, and the treatment reporting scheme (see section 1.7.1), we assumed that bath treatments were applied to all cages on the farm. In most cases, a single farm contributed more than one treatment during the study period.

### 3.3.2. Descriptive statistics

We calculated the mean, median, 90% range and number of observations (treatments) for the adult lice mean abundance one week after treatment for each level of the predictors included in the study (Table 3.1). For continuous predictors, we estimated the association with outcome using a non-parametric correlation coefficient (Spearman's $\rho$). We also estimated descriptive statistics (mean, median, standard deviation, maximum and minimum) of the adult lice mean abundance by each of the 54 salmon farming neighbourhoods as defined by the Subsecretary of Fisheries and Aquaculture (Subpesca, 2011), in order to

appreciate the spatial distribution of treatment performance (Appendix 3.2, table 3.5). For descriptive purposes we calculated the overall efficacy[2] of pyrethroids for the adult lice group.

#### 3.3.2.1. Predictors

Selection of predictors was based on a causal diagram (Figure 3.1) representing our *a priori* knowledge of the factors that impact treatment performance; the variables involved in the diagram are discussed below. Figure 3.1 is divided in two sections. One of them includes variables at the neighbouring farms that affect post-treatment adult lice levels at the farm of interest; and another containing on-farm factors that impact the treatment outcome. Among the latter, we depicted measured (e.g. pre-treatment adult lice level) and unmeasured (e.g. pre-treatment copepodid level) sea lice levels one week before treatment, and fish- and treatment-related factors during the procedure.

**On-farm variables**

Among the on-farm predictors, we included the juvenile and adult lice mean abundance observed one week before the treatment because it has been observed that pre-treatment levels significantly impact the performance of immersion treatments (Chapter 2). Mean lice abundances were calculated for the 40-fish samples. We also included fish-related variables, such as mean weight (kg), total biomass (tons), stocking density (kg of fish per $m^3$), and species (Atlantic salmon or rainbow trout), as these have been described as having an impact on *C. rogercresseyi* levels (Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013; Chapter 2). Fish-related variables were also estimated from the 40-fish sample. The week of the production cycle was included to control for trends in sea lice response to treatment over the duration of the production cycle that are not accounted for by the pre-treatment sea lice level or fish-related variables.

[2] Efficacy = (lice level 1 week pre-treatment – lice level 1 week post-treatment) / lice level 1 week pre-treatment

With respect to the treatment event, we evaluated the time (in days) that the procedure took to treat all pens in the farm. We also included the time (in weeks) since the previous treatment, because recent treatments could select for tolerant lice and, thus, undermine the current treatment's success.

Because in Chapter 2 we found some evidence for the fact that previous treatments may impact the performance of the current treatment, we included a 6-level categorical variable indicating previous treatments during four weeks before the treatment in evaluation. Categories were: no treatment (reference level), azamethiphos, pyrethroid, emamectin benzoate, diflubenzuron and mixed treatments.

**Environmental variables**

Water temperature and salinity were also included as they are known to impact development and survival of adult *C. rogercresseyi* (González & Carvajal, 2003; Bravo et al., 2008a; Bravo, 2010). Furthermore, several studies have shown that these factors are spatially structured (Dávila et al., 2002; Acha et al., 2004; Tello & Rodríguez-Benito, 2009) and, thus, may influence the level of spatial clustering.

**Off-farm variable**

Research presented in Chapters 2 and 4 in this thesis suggest the external infectious pressure (e.g. number of sea lice in neighbouring sites) significantly affects the result of immersion. In order to account for that effect on the post-treatment adult lice level we included in our model the reproduction potential of the neighbouring farms (NRP) within a 30 km seaway distance of the farm of interest, two weeks before the treatment event. Briefly, NRP considers the number of gravid females in neighbouring farms (excluding farms rearing Coho salmon), weighted by the distance between the neighbour and the farm for which NRP is calculated. Details for calculation of NRP are given in Chapter 4, section 4.3.6.1. By including the number of gravid females at neighbouring farms two weeks before the treatment we intended to control for the level of copepodids and early chalimus stages on fish (which normally are not observed on fish given their small size) before the treatment (see Figure 3.1).

**Calendar time**

Our study was performed over 87 weeks. During that period of time adult lice abundance did not show any evident seasonal behaviour; however, between weeks 58 and 70 (February and May 2013) there was a clear increment of sea lice levels. Because the time the treatment was performed may influence the treatment outcome, we included calendar time in the model building process. We assessed this predictor in linear, quadratic and cubic forms, in order to capture sea lice variations over time (see Figure 3.7b).

### 3.3.3. Multivariable analysis

We built a linear mixed model to account for the factors that influence the treatment performance. The outcome for this model was the adult lice mean abundance one week after treatment calculated based on the 40-fish sample. Adult lice included female (gravid and non-gravid), and male adult lice. We chose adult *C. rogercresseyi* for the outcome because pyrethroids target this life stage (SAG, 2013). The adult lice mean abundances were log transformed as suggested by a Box-Cox analysis, in order to better meet model assumptions. In order to permit the log transformation of zero values, we added 0.3 to the adult lice abundances. This value was chosen from a range between 0.0001 to 1, using a Box-Cox procedure as suggested by Venables & Ripley (1999).

Because, in most cases, we had several treatments per farm, we included farm random effects in order to estimate each farm's contribution to the outcome. The model equation for our final model could be expressed as:

$$\ln(Y_g + 0.3) = X_g\beta + Z_g b_{farm(g)} + \varepsilon_g$$

where $Y_g$ is the adult lice mean abundance at treatment ($g$), $X_g$ is the vector for fixed effects, $\beta$ is the corresponding coefficient vector, while $Z_g b_{farm(g)}$ represents all random terms at the farm level, including

the random intercept. Errors ($\boldsymbol{\mathcal{E}_g}$) were assumed to follow an exponential correlation structure due to repeated observations in time on each farm, that were not evenly spaced.

#### 3.3.3.1. Model building and model validation

We initially built a linear mixed model including company and farm as random effects. Random effects were kept only if the variance estimate was different from zero, based on full maximum likelihood (ML) estimation. Inference for fixed effects was based on the Wald test and for random effects we used the likelihood ratio test (LRT). Variables with the highest *p*-value were dropped one at a time, until all predictors were significant at $p<0.05$. Non-significant potential confounders ($p>0.05$) were kept in the model if their removal induced a change of 20% or greater on any other predictor's coefficient. Collinearity was reduced by retaining one predictor in cases where two or more were highly correlated ($|\rho|> 0.7$). When no further variables were dropped from the model, we tested biologically-plausible two-way interactions between fixed effects. In addition, predictors that were considered to potentially affect the farm effect were included as random slopes at the farm level. A random slope allows the coefficient of a particular predictor to vary across units of a grouping level (e.g. farms), so it captures extra variability at that level. Assumptions of normality and homoscedasticity of the random effects and residuals were explored visually using Q-Q plots and plotting standardized residuals vs. fitted values. In addition, residuals were plotted against continuous predictors to assess the assumption of linearity. Extreme observations (standardized residuals numerically > 3) were excluded in a sensitivity analysis from the final model in order to evaluate their impact on the coefficients; however, they were considered in the subsequent analyses. Statistical modelling was performed with the statistical package Stata, version 13 (StataCorp LP).

### 3.3.4. Spatial and spatio-temporal analysis

The spatial cluster analysis was performed on farm effect predictions from the model above, estimated as best linear unbiased predictors (BLUPs). Spatial clustering was evaluated using both global and local

clustering methods. In addition, a spatio-temporal analysis was performed on the residuals at the treatment level. In this case, clustering was evaluated with local methods. Global methods evaluate whether spatial clustering is present in the study area; however, they do not identify the location of clusters, while local methods identify the actual location and extent of the clusters (Pfeiffer et al., 2008).

#### 3.3.4.1. Global cluster analysis

Global cluster analysis were performed on farm effect predictions estimated from the final (full) linear mixed model, which included calendar time and production time. Global clustering of the farm effects was quantified by the Moran's *I* statistic (Pfeiffer et al., 2008), in OpenGeoDa software version 1.2.0 (www.geodacenter.asu.edu). Moran's *I* was calculated from distance weights based on non-Euclidean (i.e. seaway) distances, and it was estimated for each of five 10-km-wide distance bands (i.e. 0 to 50 km) in order to explore the extent of the spatial clustering. The hypothesis of no clustering was tested through Monte Carlo hypothesis testing based on 999 permutations of the farm effect predictions across farms in our study area. The Moran's *I* coefficient was calculated as follows:

$$I = \frac{n}{\sum_i \sum_j \omega_{ij}} \frac{\sum_i \sum_j \omega_{ij} x_i x_j}{\sum_i (x_i)^2}$$

where $x_i$ and $x_j$ are the farm effect predictions at two different locations, and $\omega_{ij}$ is a weight based on the seaway distance between locations *i* and *j*. Mean of both $x_i$ and $x_j$ were assumed equal to zero.

In models including random slopes the variance is not constant, but rather a function of the fixed predictor for the random slope (Dohoo et al., 2009). In our context, this means variance of farm effects may change along the production cycle and, therefore, the cluster detection could be affected. In order to explore this possibility we estimated Moran's *I* coefficients at week 10 and 60 in the production cycle, in addition to the analyses described in the previous section corresponding to production cycle week 34 (the mean

value)[3].

### 3.3.4.2. Local cluster analysis

Local cluster analyses were performed in order to detect clusters of high values for both farm effect predictions and residuals (e.g. treatment level data) using the Normal model of the scan spatial analysis (Kulldorff, 1997) implemented in the package SaTScan, version 9.1.1. (www.satscan.org) and based on non-Euclidian distances as described in Kulldorff et al. (2009). Clusters of low values for farm effects and residuals were also explored.

**Farm level predictions**

We conducted a purely spatial analysis for the farm level predictions limited to a spatial window up to 40 km from the farm of interest, as previous research has found *C. rogercresseyi* might spread up to 30 km (Kristoffersen et al., 2013). Farm predictions from of a "null" model (containing only the fixed effects for production week and calendar time, as well as the farm level random intercept and slope) and from the full model were computed in order to investigate if the predictors included in the final model would explain the spatial clusters observed from the null model. We use the term "null" model because it lacks all the predictors related to sea lice abundance contained in the full model (see section 3.4.2). We also performed a local cluster analysis for the random coefficients from the full model.

**Residuals**

Residuals at the treatment level were estimated from a reduced model defined as final model (as described above) without calendar time, and for a null model (as above, but also without calendar time). We excluded calendar week from these analyses in order to leave the effect of calendar time within the residuals, so a spatio-temporal analysis can be used to capture potential interactions between space and

[3] For the sake of simplicity we use the term 'farm effect prediction' for the farm effects predicted at the mean value of the production cycle week (i.e. 34). Farm effects predicted at other values of the production cycle (i.e. 10 or 60), are explicitly indicated.

time as observed in the descriptive analysis. The calendar time was divided in 21 months (January 2012 to September 2013); the spatial window was again limited to 40 km seaway distance, while the temporal window was set at 20% of the total time.

## 3.4. Results

### 3.4.1. Descriptive analysis

A total of 1,090 treatment events met the inclusion criteria of our study. These treatments were performed on 218 farms, spread over 45 neighbourhoods, 16 from the Los Lagos region, and 29 from the Aysén region (Figure 3.2). The number of farms per neighbourhood averaged 4.8, ranging between 1 and 10. There were, on average, 5.0 treatments per farm (range: 1-17). Eighty-eight percent of treatments were performed on Atlantic salmon, while 12% on rainbow trout. The average weight of Atlantic salmon and rainbow trout was 2.03 kg (SD=1.21) and 1.73 kg (SD= 0.71), respectively. During the study period the water temperature averaged 10.8° C (SD=1.4).

The number of adult *C. rogercresseyi* one week after a pyrethroid treatment had an overall mean of 6.9 (SD=14.8) lice per fish, although it varied considerably across farms (Figure 3.2 and Table 3.5). Very high levels of lice after treatment (> 40 adult lice) were observed in both regions, and particularly in the areas of Calbuco, central Chiloé, and in north-eastern and central Guaitecas, corresponding to neighbourhoods 3a, 16, 18a, 18d, 20 and 24. Consistently, treatment outcomes in some of these neighbourhoods had a large coefficient of variation (CV > 2) (Table 3.5). Lower counts (mean < 3 adult lice/fish) were observed all across the study area, but they were more frequent in the Los Lagos region. The adult lice mean abundance one week before the treatment was 8.7 (SD=11.2). The mean efficacy of pyrethroids on adult lice stages was 0.18 (SD=0.98). Salmon farming neighbourhoods are presented in Figure 3.6.

Associations between predictors and our outcome were appreciable (Table 3.1). Among the on-farm predictors, pre-treatment sea lice levels exhibited a positive relationship with the levels of lice after the

procedure. We also observed a positive association pattern between our outcome and fish-related variables, such as weight and fish biomass. The duration of the treatment procedure also showed positive relationship with post-treatment sea lice levels. The salmon species showed a positive association with the post-treatment levels, with rainbow trout showing a slightly greater mean abundance than Atlantic salmon.

The post-treatment adult lice levels seemed to increase as time goes by in the production cycle (Table 3.1 and Figure 3.7a). This pattern may be the result of the high correlation between production cycle week and both fish weight and fish biomass (0.88, $p<0.001$, and 0.79, $p<0.001$, respectively). The Spearman correlation coefficient ($\rho$) between the adult lice abundance and production cycle week was 0.44 ($p<0.001$). Calendar time also appeared to be associated with the post-treatment sea lice levels ($\rho=0.37$, $p<0.001$) (Figure 3.7b). In the case of environmental variables, water temperature exhibited a low, but significant positive association with our outcome ($\rho=0.08$, $p<0.011$), while water salinity showed no relation with the post-treatment levels ($\rho=-0.01$, $p=0.638$).

Finally, the number of neighbouring farms within a 30 km seaway distance showed a positive pattern with our outcome, but only at low and medium values of adult lice levels.

### 3.4.2. Multivariable analysis

#### 3.4.2.1. On-farm variables

Out of 9 on-farm predictors we identified for the model building process, only four were kept in the final model. We found that the sea lice level one week before the treatment was positively associated with the adult lice level one week after the procedure. This relationship was significant for pre-treatment levels of both adult and juvenile lice ($p<0.001$) (Table 3.2).

Our model estimated that the increment of adult lice along the production cycle week was at an average rate of 14% ($p<0.001$) per 10 weeks (Table 3.2). The inclusion of a random slope significantly improved the model fit, suggesting that the increment of sea lice along the production cycle significantly varied across farms. In 95% of cases, the rates were between 7% lower lice per 10 weeks and 39% higher lice per 10 weeks. A plot for the predicted farm slopes for production cycle week shows graphically this variability (Figure 3.12).

It is important to note that the production cycle week, mean fish weight, and fish biomass were highly correlated (correlations ranged between 0.72 and 0.80). Models containing production cycle week exhibited a better fit than models with either mean fish weight or fish biomass; therefore, the former was chosen in the final model.

Regarding the treatment procedure, the time it took to complete the treatment for the whole-farm was also positively associated with the outcome. In particular, the model showed that per each extra day required to complete the treatment, the adult lice increased by 3.4% ($p<0.001$). Previous delousing treatments in the same farm during 4 weeks before the treatment in evaluation did not have a significant impact ($p=0.099$) on the adult lice levels.

On-farm predictors such as fish stocking density, salmonid species, and time since previous treatment did not have any significant effect on the outcome ($p=0.686$, $p=0.557$, and $p=0.279$, respectively), and therefore were removed from the final model.

#### 3.4.2.2. Environmental variables

Among environmental variables, only water salinity was retained in the final model. Within the salinity range of our dataset (12 to 36 ppt) this predictor had a significant positive association with the outcome ($p<0.001$), increasing the adult lice level one week after treatment by 4.6% for each extra unit of water

salinity. On the other hand, water temperature did not show a significant association with the post-treatment adult lice levels ($p$=0.224).

#### 3.4.2.3. Off-farm variables

We found that the external infectious pressure two weeks before treatment, expressed as the neighbouring farms' reproduction potential in a 30 km seaway distance (NRP), had a positive and significant effect ($p$<0.001) on the adult level one week after treatment. An increase of NRP by one unit (which can be, for example, similar to increasing from no neighbouring farms to having four neighbouring farms at 2.5 km, each with 10 gravid female lice, on average) impacted the adult lice level by 34%.

#### 3.4.2.4. Calendar time

The association between calendar time and the post-treatment adult lice levels was highly significant. The quadratic form of this predictor fitted the data best. Model predictions of adult lice abundance along calendar time (setting the rest of predictors at their mean values) show that the sea lice level increased steadily from the beginning of the study period until week 70, and then it dropped slightly to week 87.

#### 3.4.2.5. Variance components

In the models, the unexplained variance remained at both the farm and treatment levels. The variance at the farm level was represented by the farm random effect and the random coefficient of the cycle production week. The proportion of the variance at the farm level (i.e. intra-class correlation coefficient, ICC) varied as a function of the production cycle week. Higher values of ICC were reached at the start and finish of the cycle (ICC start: 0.49; ICC finish: 0.43), while the lowest value was around the middle of the cycle (ICC 0.25) (see Figure 3.11). At an early stage in the model building process, the analysis showed that company variance was zero and had no effect on the outcome, therefore, from then on all analyses only included farms as random effects.

We did not notice any substantial improvement of the model fit when the exponential residual structure was included; therefore, we assumed the autocorrelation of residuals was explained by the farm level random slope and no further modeling of the dependence between residuals was needed. Hence, residuals were assumed to be independent in the final model.

#### 3.4.2.6. Model fit

Residuals at the treatment level were approximately normally distributed (Figure 3.10a). Only 4 (0.4%) of the observations had standardized residuals greater than numerically 3. In addition, residuals appeared to be homoscedastic as similar variances were observed across fitted values (Figure 3.10b). Assumption of linearity between the outcome and continuous predictors was met.

Predictions for the farm effect, as well as random coefficients, did not show any obvious departure from normality and appeared to be homoscedastic (Figures 3.8a, 3.8b, 3.9a, and 3.9b).

### 3.4.3. Spatio-temporal analysis

#### 3.4.3.1. Global cluster analysis

The Moran's *I* indicated that the farm effect predictions from the full model (estimated at week 34 in the production cycle) exhibited a positive spatial autocorrelation and this correlation depended on the distance band used (Figure 3.3). Moran's *I* coefficient observed in the 0 to 10 km distance band for weeks 10, 34 and 60 were 0.326 ($p$=0.001), 0.267 ($p$=0.001) and 0.086 ($p$=0.127), respectively. This means that farms with more or less lice than expected after treatment (based on the predictors of the final model) were located closer together in space, but only during the first half of the production cycle. In the second half, treatment outcomes were no longer similar even between farms located in the proximity. Generally, the similarity among treatment performances appeared to diminish as distance between farms increased; however, this trend was not linear over distance bands (Figure 3.3).

#### 3.4.3.2. Local cluster analysis

**Farm level predictions**

The purely spatial scan statistic performed on the farm effect predictions from the null model found two significant clusters of high values. The most significant cluster (cluster 1, $p$=0.001) was located in central Aysén region, including all farms from neighbourhood 20 and farms in neighbourhoods 18b, 18c, 18d, 19b and 21b (Figure 3.4a). This cluster consisted of 29 farms. The mean adult lice level at farms within this cluster was 1.55 times greater than in the rest of the study area (Table 3.3). The second significant cluster (cluster 2, $p$=0.002) consisted of 4 farms located in the neighbourhood 3a, in northern Los Lagos region (Figure 3.4a). In this case the, the relative ratio of adult lice counts was 2.79:1 in favour of farms within the cluster (Table 3.3).

When we ran the local cluster analysis on the farm effect predictions from the full model, practically the same two significant clusters were found; however, the cluster 1 ($p$=0.011) expanded to the west including new farms from neighbourhoods 19a, 19b and 20, but excluding farms from neighbourhoods 18c and 18d in the east (Figure 3.4b). Adult lice levels inside the cluster were 1.22 greater than those outside the cluster. This cluster included 28 farms. The cluster 2 ($p$=0.024) included the same four farms as the analysis based in the null model; the relative ratio in adult lice counts decreased to 1.60:1 (Table 3.3). No significant clusters of low values for farm effects or for random coefficients were detected (results not shown).

**Residuals**

The spatio-temporal analysis performed on the residuals from the null model detected three significant clusters. In these areas, the observed sea lice counts after the treatment were higher than predicted by the final model (Figure 3.5 and Table 3.4). The most significant cluster was located in neighbourhood 18a (northern Aysén region), during March, April and May 2013, and consisted in 7 farms. During that period of time, adult lice levels inside the cluster were 3.69 times greater than in the rest of the study area. The

second significant cluster ($p$=0.002) was observed in central Aysén region between February and April 2013, and involved 16 farms from neighbourhoods 20, 19b, 19a and 21b. In this case, the relative ratio of adult lice counts inside and outside the cluster was 1.94:1. A third significant cluster ($p$=0.041) was detected in northern Los Lagos region during March and April 2013, and consisted in four farms from neighbourhood 3a. Adult lice levels inside the cluster were 2.57 times greater than outside the cluster. When the same analysis was carried out using the residuals from the full model, these three clusters were no longer significant, and no other clusters were found. No significant clusters of low values for residuals were detected.

### 3.5. Discussion

Our analysis demonstrated some clustering of the performance of pyrethroids treatments in space in Chile between January 2012 and September 2013. When the spatial clustering was analysed at the farm level (farm effect predictions), we found two significant clusters: cluster 1 in central Aysén region (neighbourhoods 18b, 18c, 18d, 19b, 20 and 21b) and cluster 2 in northern Los Lagos region (neighbourhood 3a) (Figure 3.4a). This agrees with the descriptive analysis, where it was observed that the worst treatment results were in these neighbourhoods (Figure 3.2). These two clusters remained when we controlled for on- and off-farms and environmental variables (Figure 3.4b), suggesting that other unmeasured factors were driving the spatial dependence of treatment performance across farms.

The spatio-temporal analysis performed on residuals at the treatment level revealed three clusters, two in the same area as the clusters detected in the purely spatial analysis, plus a third one located in northern Aysén region (Figure 3.5). These three clusters were no longer significant after controlling for all predictors in the full model. This suggests that the clustering was driven by the predictors included in the final model, and that these predictors clustered in space and time as well.

### 3.5.1. Global cluster analysis

The global spatial cluster analysis performed on the farm effect predictions from the full model found that treatment outcomes were more similar at shorter distances between neighbouring farms. Because the models accounted for the most important predictors, these findings might be the result of tolerance development of sea lice to pyrethroids, or due to factors related to the treatment procedure. It is important to mention that we did not have access to any information related to sensitivity of sea lice to pyrethroids (i.e. bioassay results), or details regarding the treatment modality (i.e. tarpaulin, well boat) or drug administration; therefore, their potential effects remain in the model's unexplained variability (i.e. farm effects and residuals).

In Chile, low sensitivity of *C. rogercresseyi* to pyrethroids has been reported in the Los Lagos region (Helgesen et al., 2014); however, there are no studies assessing the geographical variation of sea lice sensitivity to pyrethroids. Research performed in Canada have shown that sensitivity of *L. salmonis* to emamectin benzoate varies in space (Whyte et al., 2013). It is well known that spread of resistance to chemotherapeutants in sea lice is mediated by the flow of resistance genes between sea lice metapopulations (i.e. farms) (Denholm et al. 2002; Tully & Nolan, 2002). It is, therefore, logical to expect that farms located closer together may share similar resistance genes, have similar levels of sensitivity to chemoterapeutants, and therefore, have similar treatment responses.

We also found that the similarity of treatment performance in space depended on the stage of the production cycle when it was evaluated. Specifically, the treatment performance clustered in the first half of the production cycle (i.e. weeks 10 and 34), but not in the second half (i.e. week 60). Consistently, the scan spatial statistic found two significant clusters at week 10 in the production cycle, but none at week 60 (data not shown). This trend was observed in the full model which contained calendar time suggesting that treatment outcomes were similar in space at a particular week of the production cycle, regardless of the actual time at which the cycle was.

The lack of spatial clusters towards the end of the production cycle weakens the hypothesis of sensitivity to pyrethroids as the driver of the spatial dependence among farm effect predictions, because resistance should be maintained and even increased in the sea lice population over time. However, it may also indicate that other factors play a role in the observed spatial variability, making the treatment performance more dissimilar among neighbouring farms later in the production cycle. Such factors should most likely to be related to the age or size of fish, because these variables behave in a similar manner across farms, regardless of the calendar time.

A possible explanation for the lack of clustering in the second half of the production cycle is that as the production cycle progresses the fish weight increases, and this variable has been associated with higher levels of *C. rogercresseyi* (Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013; Chapter 2). The problem with this explanation is that our full models corrected for this effect by including the pre-treatment sea lice levels. Another more plausible explanation is that, as the production cycle progresses and fish gain weight, the likelihood that immersion treatments are not properly carried out increases. This happens because the net is rarely raised to the optimum height to reduce the water volume with larger fish, and the exposure time with the drug solution might be reduced as oxygen is rapidly consumed under these conditions (S. Bravo, pers. comm.). Because the level of compliance of treatment procedures is inherent to the farm, this may lead to different treatment outcomes among neighbouring farms with heavier fish. In support of this hypothesis, we observed that in the farms included in the two significant clusters, the variability (measured by the simple standard deviation, SD) of the farm effects increased as time went by, and largely surpassed the overall SD in the second half of the production cycle. In contrast, the farm effect SD within the clusters was lower than the overall SD during the first half of the cycle, showing the treatment performances to be more similar across farms at that time (see Figure 3.13). This suggests that the similarity of farm effect predictions in an area was important for detecting significant clusters.

Our final (full) model showed that the sea lice trends over the production cycle varied significantly across farms, ranging from marked increase to light decrease of lice levels. If we consider that in the final model we controlled for different external and internal infectious pressures, and environmental factors as well, differences in on-farm production conditions remain as potential drivers for such patterns. Some production factors that showed an important variability in our dataset and that have been demonstrated to impact sea lice levels are number of stocked fish, fish biomass, fish mean weight and stocking density.

### 3.5.2. Local cluster analysis

Generally speaking, the purely spatial and the spatio-temporal analysis could be picking up two different components of clustering. The spatial analysis based on farm effect predictions represents unknown factor(s) of slow development or somewhat constant in time, while the spatio-temporal analysis performed on residuals is related to unknown factor(s) which vary between treatments within a farm.

It is worth noting that when we controlled for predictors (full models), clustering of farm effect predictions was marginally affected (i.e. the same number of clusters between the null and full models were found), while clustering for residuals disappeared completely. This suggests that the predictors mostly explained factors operating at the treatment level, which seems logical since the majority of the fixed effects included in the final model (with the exception of water salinity) showed a larger variation between treatments compared to between farms (see farm ICC in table 3.1). Consequently, it can be presumed that farm level predictions are representing farm level factor(s) more constant in time. Furthermore, we learnt these farm effect predictions were clustered in space. A potential factor that fits the behaviour of farm level predictions is the resistance of sea lice to pyrethroids.

It is interesting to observe the different effect that controlling of predictors had on the two clusters detected in the spatial analysis. When we controlled for predictors in the purely spatial analysis, cluster 1 expanded to a larger area, instead of shrinking as it would be expected, although the total number of farms

reduced from 29 to 28. We think that happened because the new farms included in the cluster 1 with the full model were more distant, and SaTScan does not take into account the actual seaway distance between farms, but a rank of "closeness". In the case of cluster 2, controlling for predictors did not have any impact on the size/location of the cluster. This may have happened because farms within cluster 2 were relatively isolated from the rest of the farms in the area (Figure 3.4b)

The three clusters detected in the spatio-temporal analysis (based on the null model) were consistently present during the last months of the farms' production cycles. Descriptive data shows that post-treatment lice levels increased at the end of the production cycle (Table 3.1), and, in many cases, these peaks occurred at similar calendar time, which seems logical due to coordination of the production cycle within neighbourhoods in Chile. When we controlled for pre-treatment levels and external infectious pressure (NRP), these clusters disappeared which suggests these variables accounted for exchange of sea lice between neighbouring farms. In other words, the spatio-temporal clusters observed in the null model were due to sea lice exchange at the end of the production cycle, and they were more prone to be detected in areas where the production cycles were coordinated in time.

Other unknown factors that may impact sea lice levels among the last months of the production cycle, such as the level of compliance of treatment procedures, might also explain this variation; however, as clustering disappeared after controlling for predictors, we can presume these unknown factors would not show spatial dependence among farms. This is consistent with our interpretation of results from the spatial cluster analysis (farm effect predictions), because these unknown factors that could explain the lack of cluster in the spatio-temporal cluster analysis, may be the same as those responsible for the lack of clustering in the second half of the production cycle (purely spatial analysis).

### 3.5.3. Study limitations

We excluded from our analysis pyrethroid-based treatments with durations of greater than 16 days. In

general, longer treatments occur when a second drug is applied (i.e. oral treatment), which may be a sign of low efficacy of pyrethroids in a particular farm. This may have introduced selection bias in our study; however, if the excluded farms had indeed poorer treatment performance, the (spatial) variation of treatment performance found in our study might have been underestimated (i.e. bias towards the null).

We did not consider the method by which the pyrethroid was administered. At the time of the study, there were three methods used in the industry: tarpaulin, skirt, and well-boat. There is some anecdotal evidence that tarpaulin and well-boats are more effective at delivering treatments (i.e. lower post-treatment sea lice levels) than skirt (R. Ibarra, pers. comm.). Because treatment modalities may change between treatments within a farm, it is unlikely that the farm's random effect have fully captured its effect, consequently, this may have biased our spatial cluster analysis. On the contrary, the spatio-temporal analysis could not have been affected by this lack of information as residuals at the treatment level may have incorporated this variability.

The short time frame defined for this study (relative to the salmon production cycle), resulted in that, in some cases, we were able to include only a portion of the production cycle. That may affect the cluster detection especially in the spatio-temporal analysis. Future research should consider longer study periods, ideally including at least 2 production cycles in order to better explore spatio-temporal clusters.

Finally, it is important to mention that an important proportion of sea lice counts in our dataset remained below 8 lice/fish (see Figures 3.7a/b and 3.14b). This unusual pattern might due to the fact that farmers have to treat as soon as sea lice levels surpass 6 adult lice/fish, but it may also denote some level of under-reporting because farms are exposed to strong control measures, such as anticipated harvest, if adult lice levels surpasses 9 adult lice/fish for more than 3 consecutive weeks. This situation may have biased our results to some extent.

## 3.6. Conclusion

We found that the performance of pyrethroids was clustered in space and time in Chile between January 2012 and September 2103. There were two areas where the response to treatments on farms were more similar for farms in close proximity, even after controlling for environmental and management factors. The reason for the spatial clusters of poor treatment responses is unknown, but it appears that there may be areas that are more problematic for sea lice control. Further research is required to confirm the patterns observed in this study and determine their cause, including the possibility of resistance.

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## 3.8. Tables and figures

Table 3.1. Farm-level intra-class correlation coefficients (ICC) and descriptive statistics for the adult *C. rogercresseyi* mean abundance post-treatment at different levels of the predictors included in the model building process.

| Variables | Farm ICC [1] | Levels | Mean | Median | 90% range | n |
|---|---|---|---|---|---|---|
| Adult lice mean abundance one week before treatment | 0.156 | < 4 | 2.82 | 1.83 | 0.13 – 10.93 | 204 |
| | | ≥ 4 to < 8 | 4.97 | 3.95 | 0.58 – 9.05 | 507 |
| | | ≥ 8 | 11.67 | 5.90 | 1.48 – 65.18 | 379 |
| Juvenile lice mean abundance one week before treatment | 0.293 | < 2 | 2.75 | 1.95 | 0.15 – 8.08 | 303 |
| | | ≥ 2 to < 6 | 5.31 | 4.03 | 0.75 – 9.58 | 488 |
| | | ≥ 6 | 13.70 | 6.93 | 2.08 – 66.83 | 299 |
| Fish biomass within farm during treatment (tons) | 0.281 | < 1,000 | 4.68 | 2.98 | 0.23 – 9.05 | 361 |
| | | ≥ 1,000 to < 2,000 | 6.00 | 4.03 | 0.38 – 12.15 | 417 |
| | | ≥ 2,000 | 10.66 | 5.38 | 1.48 – 45.38 | 312 |
| Fish mean weight at treatment (kg) | 0.185 | < 1.5 | 4.17 | 2.88 | 0.20 – 8.78 | 423 |
| | | ≥ 1.5 to < 3 | 6.74 | 4.20 | 0.48 – 13.53 | 430 |
| | | ≥ 3 | 12.05 | 5.88 | 1.85 – 57.60 | 237 |
| Stocking density during treatment ($kg/m^3$) | 0.298 | < 4 | 3.63 | 2.88 | 0.23 – 8.58 | 366 |
| | | ≥ 4 to < 7 | 6.80 | 4.03 | 0.40 – 12.15 | 349 |
| | | ≥ 7 | 10.17 | 5.45 | 1.18 – 45.38 | 375 |
| Species | . | Atlantic salmon | 6.82 | 4.16 | 0.38 – 17.88 | 954 |
| | | Rainbow trout | 7.44 | 3.35 | 0.28 – 29.43 | 136 |
| Production cycle week | 0.141 | < 30 | 3.68 | 2.78 | 0.23 – 8.65 | 422 |
| | | ≥ 30 to < 50 | 5.59 | 4.40 | 0.73 – 11.88 | 455 |
| | | ≥ 50 | 16.05 | 6.55 | 2.03 – 68.28 | 213 |
| Calendar time (months) | 0.527 | Jan-12 – Jun-12 | 2.56 | 1.91 | 0.15 – 7.35 | 156 |
| | | Jul-12 – Nov-12 | 3.91 | 3.46 | 0.29 – 8.30 | 340 |
| | | Dec-12 – Apr-13 | 11.26 | 5.18 | 0.85 – 57.60 | 358 |
| | | May-13 –Sep-13 | 7.45 | 5.09 | 1.10 – 16.60 | 236 |
| Duration of the treatment procedure in the farm (days, limited to 16 days) | 0.202 | < 3 | 5.54 | 2.96 | 0.20 – 12.05 | 316 |
| | | ≥ 3 to < 6 | 6.99 | 4.36 | 0.58 – 15.88 | 546 |
| | | ≥ 6 | 8.56 | 4.80 | 0.83 – 32.83 | 228 |
| Time since last treatment (weeks) | 0.371 | < 5 | 8.92 | 5.10 | 0.80 – 29.43 | 479 |
| | | ≥ 5 to < 8 | 6.58 | 4.25 | 0.48 – 19.45 | 305 |
| | | ≥ 8 | 4.05 | 2.63 | 0.20 – 11.43 | 306 |
| Water temperature (° C) | 0.042 | < 10 | 4.16 | 3.73 | 0.50 – 8.60 | 321 |
| | | ≥ 10 to < 12 | 7.38 | 4.63 | 0.55 – 16.6 | 471 |
| | | ≥ 12 | 9.07 | 3.79 | 0.23 – 45.98 | 298 |
| Water salinity (ppt) | 0.850 | < 30 | 4.05 | 3.25 | 0.35 – 8.60 | 147 |
| | | ≥ 30 to < 32 | 6.76 | 4.53 | 0.78 – 19.35 | 440 |
| | | ≥ 32 | 7.85 | 3.85 | 0.30 – 22.65 | 503 |
| Number of neighbouring farms within a 30 km seaway distance, NRP | 0.034 | < 6 | 3.66 | 3.08 | 0.23 – 8.58 | 141 |
| | | ≥ 6 to < 12 | 7.72 | 3.90 | 0.28 – 26.88 | 514 |
| | | ≥ 12 | 6.97 | 4.73 | 0.75 – 18.8 | 435 |

[1] Intra-class correlation coefficient (ICC) estimated from a null linear mixed effects model with the variable as the outcome and with farm random effect as the only predictor.

Table 3.2. Coefficient estimates, standard errors and *p*-values for explanatory variables in the final model for the log adult *C. rogercresseyi* mean abundance one week after an immersion treatment using synthetic pyrethroids on farmed Atlantic salmon and rainbow trout, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile (n=1,090).

| Variable name | Estimate | Standard error | *p*-value | 95% confidence interval | |
|---|---|---|---|---|---|
| **Fixed effects parameters** | | | | | |
| Intercept | -1.6882 | 0.3577 | | -2.3893 | -0.9871 |
| Log of adult mean abundance one week before treatment | 0.2116 | 0.0365 | **<0.001** | 0.1401 | 0.2832 |
| Log of juvenile mean abundance one week before treatment | 0.2269 | 0.0261 | **<0.001** | 0.1757 | 0.2781 |
| Water salinity (ppt) | 0.0447 | 0.0105 | **<0.001** | 0.0242 | 0.0652 |
| Production cycle week (centered at 34, rescaled by 10) | 0.1289 | 0.0205 | **<0.001** | 0.0887 | 0.1689 |
| Duration of the treatment procedure in the farm (days) | 0.0333 | 0.0080 | **<0.001** | 0.0176 | 0.0490 |
| Calendar time (weeks, rescaled by 10) | 0.2362 | 0.0520 | **<0.001** | 0.1343 | 0.3381 |
| Calendar time (weeks, rescaled by 10) (quadratic term) | -0.0169 | 0.0046 | **<0.001** | -0.0258 | -0.0079 |
| Neighbourhood reproduction potential two weeks before treatment (30 km seaway distance) | 0.2910 | 0.0331 | **<0.001** | 0.2261 | 0.3559 |
| **Random effects parameters** | | | | | |
| Farm | | | | | |
| var: intercept | 0.1130 | 0.0221 | | 0.0770 | 0.1658 |
| var: beta (production cycle week) | 0.0105 | 0.0040 | | 0.0050 | 0.0220 |
| covar: intercept, production cycle week | -0.0104 | 0.0068 | | -0.0238 | -0.0030 |
| Residual | | | | | |
| var: error | 0.3121 | 0.0165 | | 0.2815 | 0.3461 |

Table 3.3. Spatial clusters of high farm effect predictions (at production week 34) from null and full linear mixed models for adult lice abundance one week after a single treatment with pyrethroids, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile.

| Model | Cluster | Number of farms | Neighb. involved | Seaway distance between the two most distant farms (km) | Log adult lice | | Relative ratio in adult lice counts (inside:outside) | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mean (SD) inside | Mean (SD) outside | | |
| Null | 1 | 29 | 18b, 18c, 18d, 19b, 20, 21b | 67.18 | 0.379 (0.339) | -0.056 (0.429) | 1.55:1 | 0.001 |
| Null | 2 | 4 | 3a | 6.21 | 1.008 (0.657) | -0.019 (0.417) | 2.79:1 | 0.002 |
| Full | 1 | 28 | 18d, 19a, 19b, 20, 21a, 21b | 76.61 | 0.176 (0.168) | -0.025 (0.249) | 1.22:1 | 0.011 |
| Full | 2 | 4 | 3a | 6.21 | 0.459 (0.214) | -0.009 (0.242) | 1.60:1 | 0.024 |

Table 3.4. Spatio-temporal clusters of high residuals at the treatment level from the null linear mixed models for adult lice abundance one week after a single treatment with pyrethroids, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile.

| Cluster | Number of farms | Months | Neighb. involved | Seaway distance between the two most distant farms (km) | Log adult lice | | Relative ratio in adult lice counts (inside: outside) | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mean (SD) inside | Mean (SD) outside | | |
| 1 | 7 | Mar-13 to May-13 | 18a | 12.18 | 1.290 (0.396) | -0.016 (0.534) | 3.69:1 | 0.001 |
| 2 | 16 | Feb-13 to Apr-13 | 20, 19a, 19b, 21b | 55.89 | 0.651 (0.609) | -0.013 (0.541) | 1.94:1 | 0.002 |
| 3 | 4 | Mar-13 to Apr-13 | 3a | 6.21 | 0.936 (0.383) | -0.007 (0.546) | 2.57:1 | 0.041 |

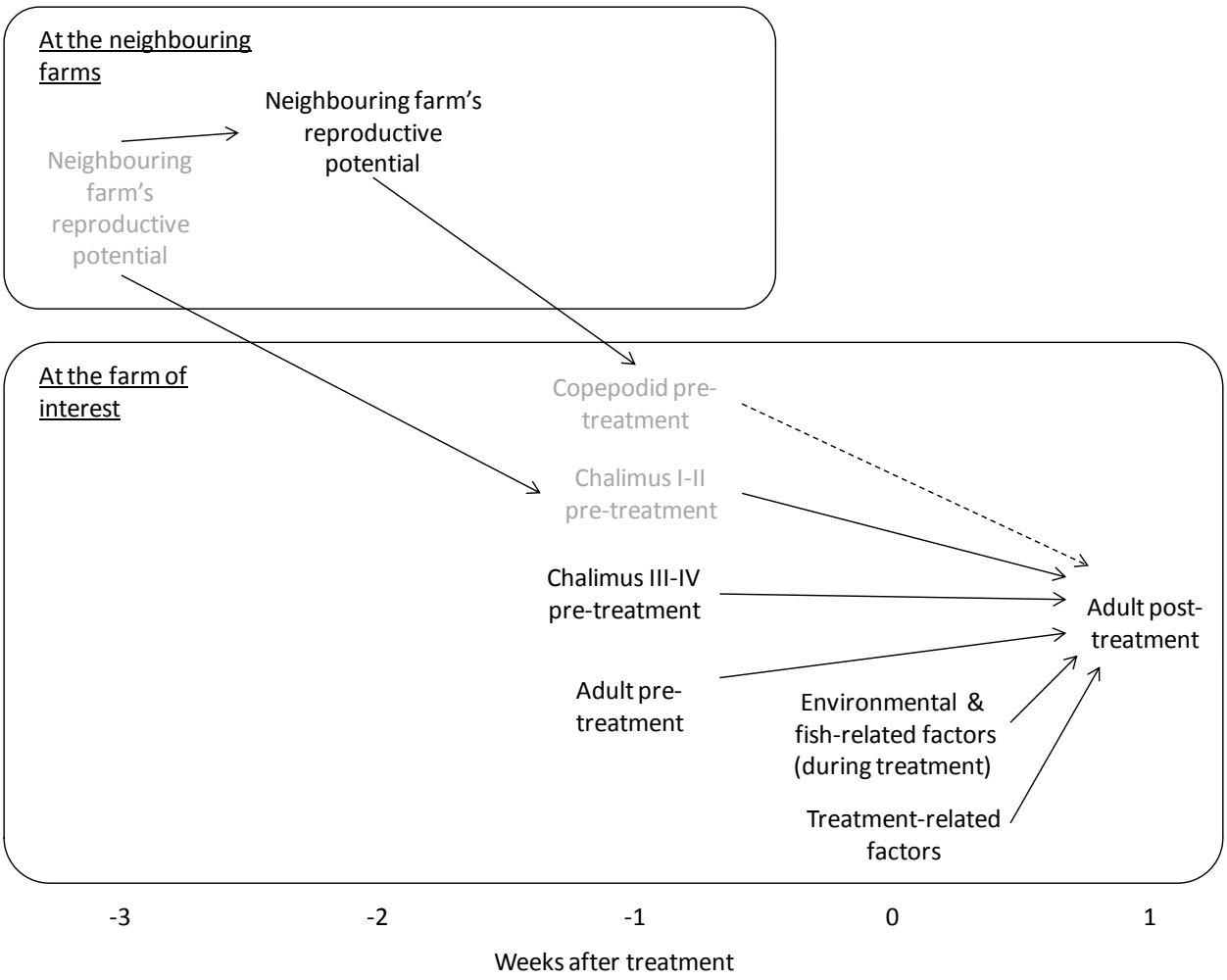


Figure 3.1. Causal diagram for treatment performance measured as adult lice mean abundance one week after treatment, for treatment duration from 1 to 16 days. Variables in black: measured; variables in gray: unmeasured (i.e. latent). Estimated likelihood of association: very likely (solid arrows) and likely (dashed arrows). Variables recorded at t=0 are detailed in section 3.3.2.1.

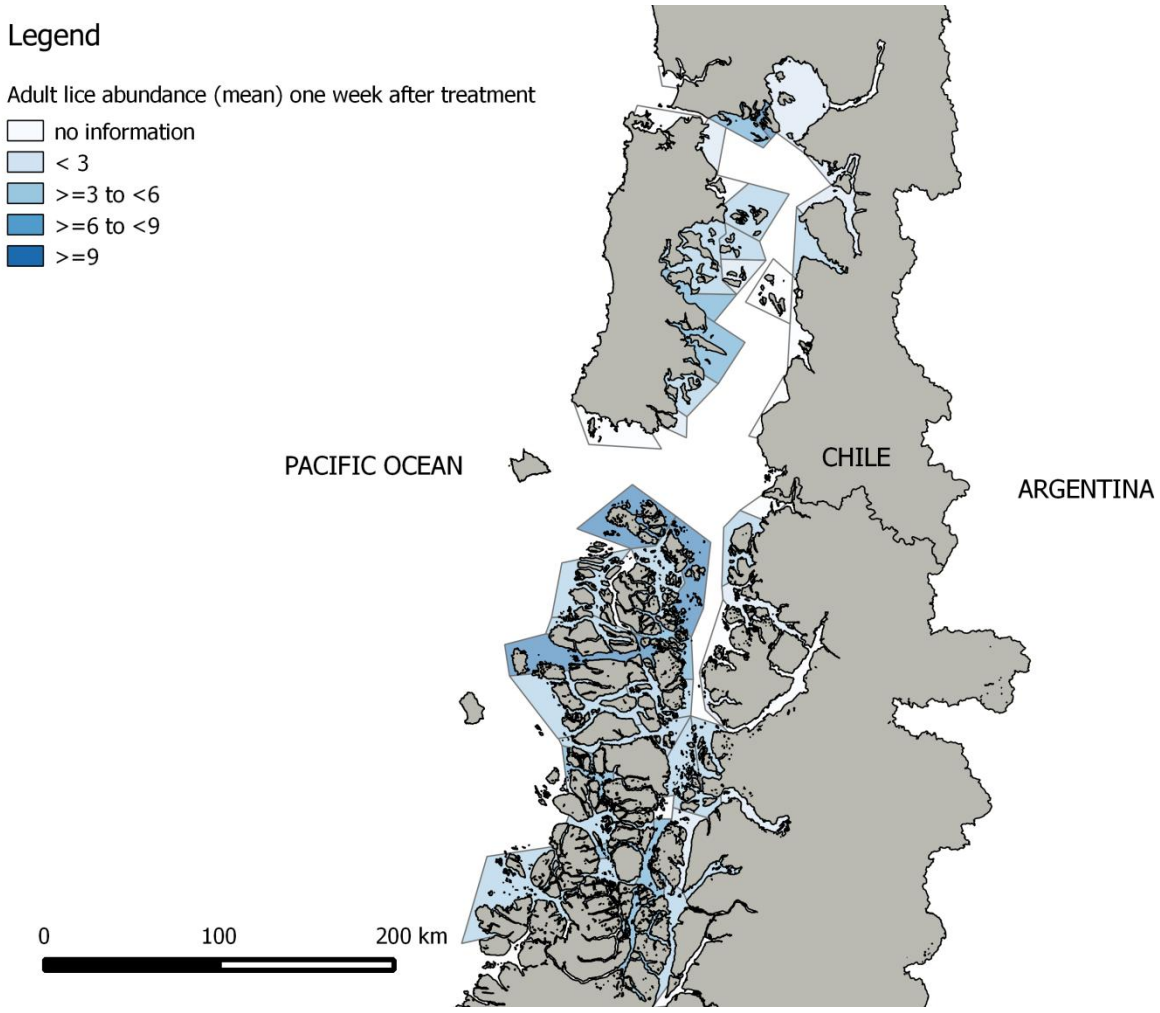


Figure 3.2. Mean abundance of adult *C. rogercresseyi* one week after treatment by salmon farming neighbourhood during January 2012 and September 2013 in the study area.

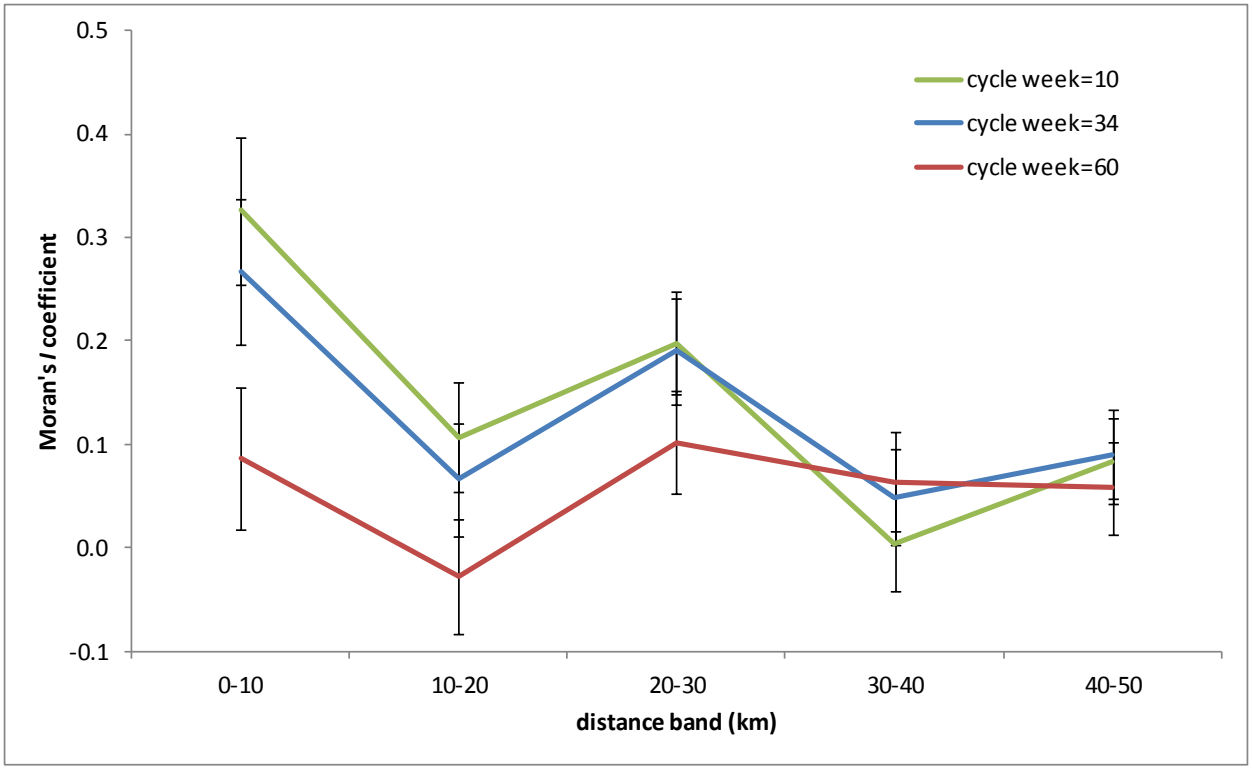


Figure 3.3. Moran's *I* coefficients with simple standard error bars for farm effects at production cycle weeks 10, 34 (random intercept), and 60 from the final model at different seaway-distance bands.

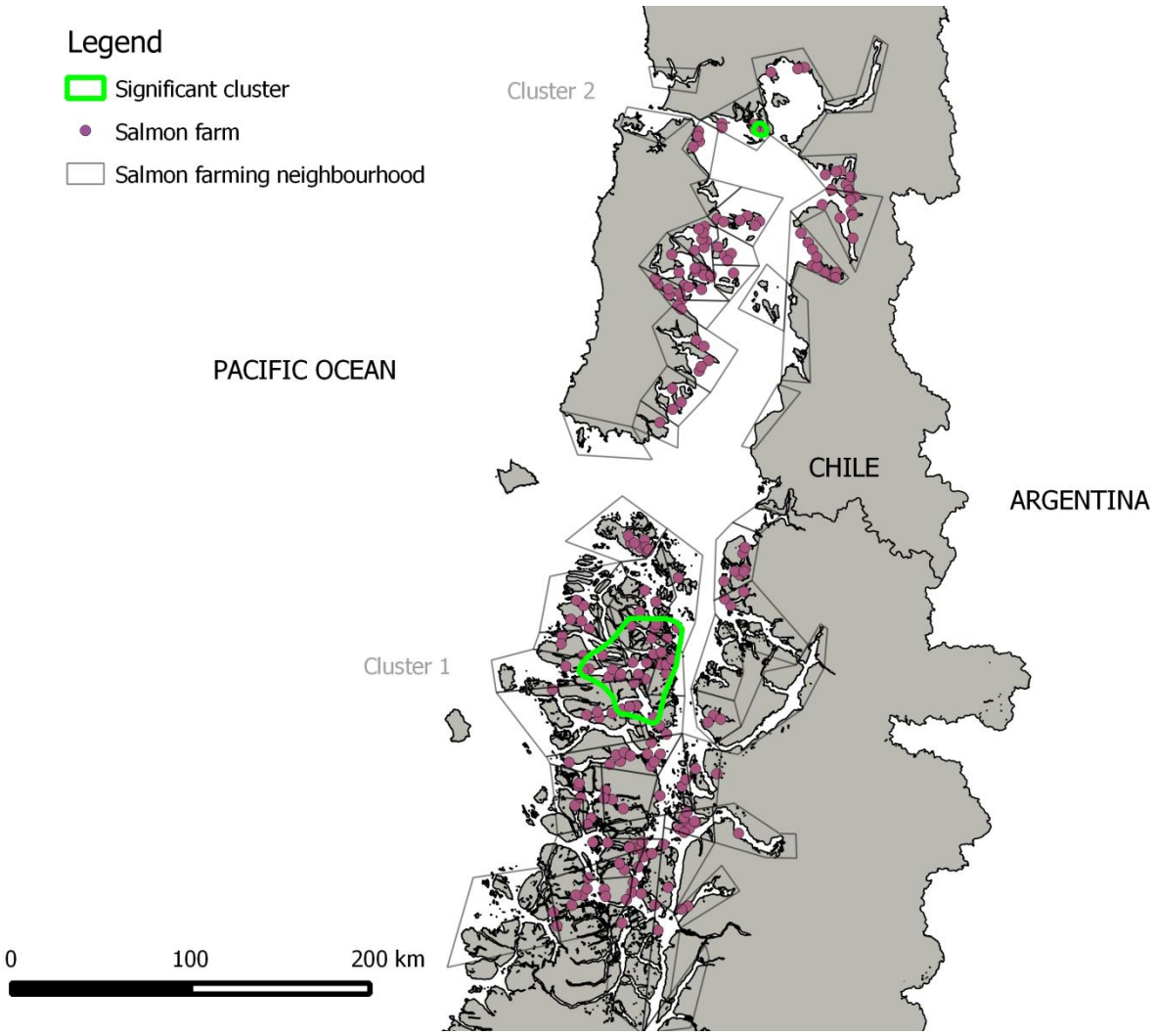


Figure 3.4a. Significant clusters of high farm effect predictions from the null model at week 34 detected in the purely spatial cluster analysis.

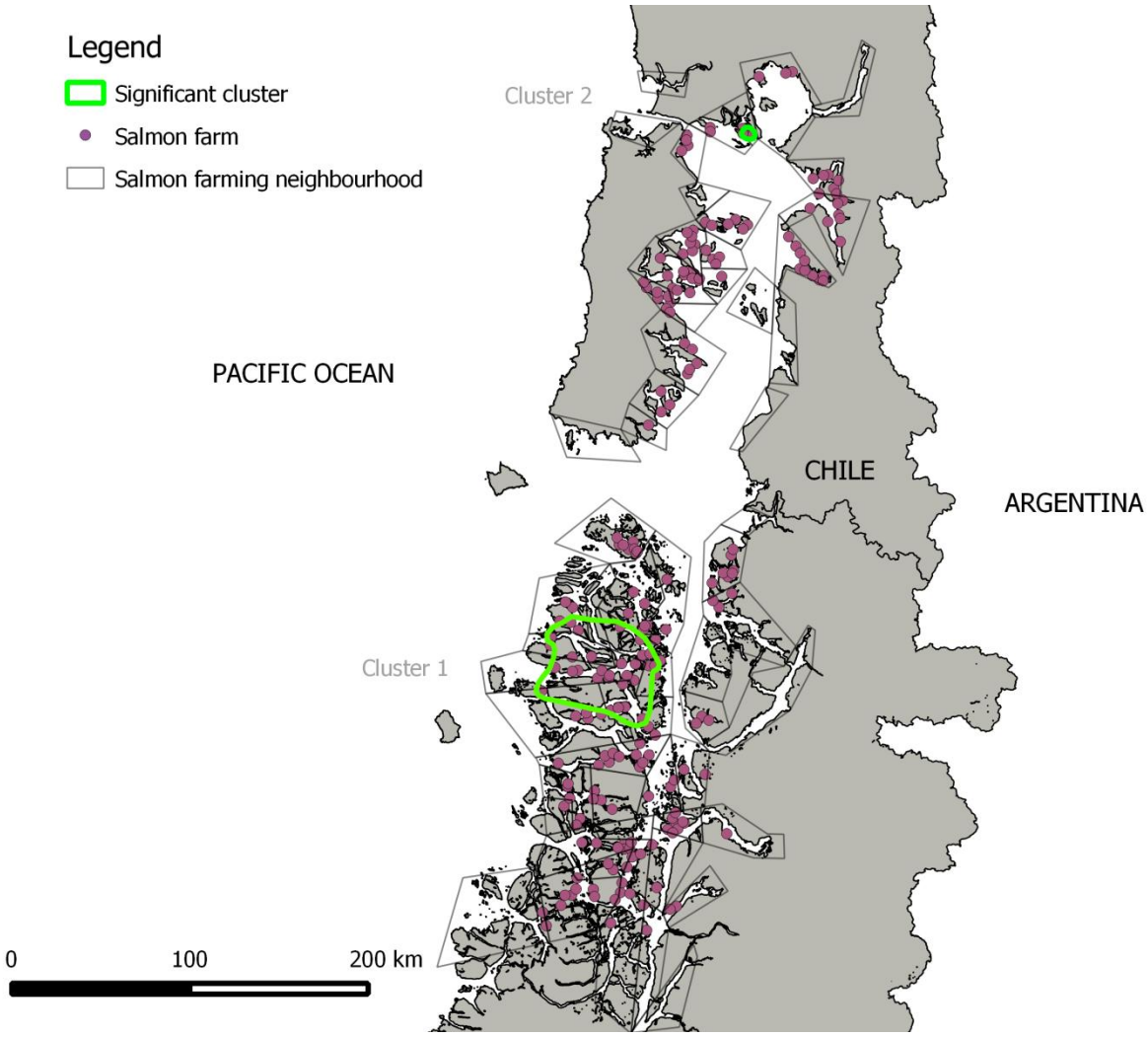


Figure 3.4b. Significant clusters of high farm effect predictions from the full model at week 34 detected in the purely spatial cluster analysis.

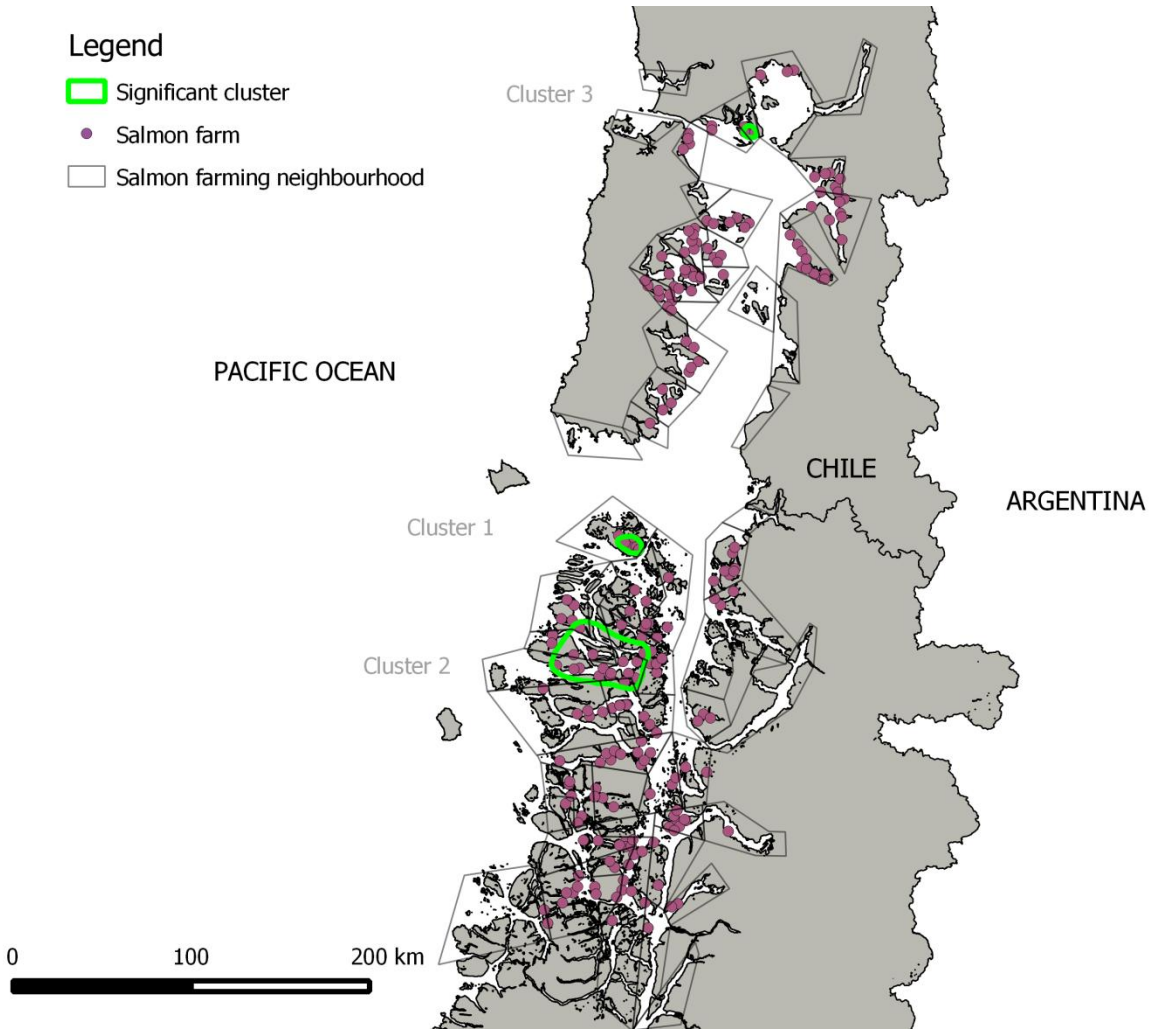


Figure 3.5. Significant clusters of high treatment level residuals from the full model detected in the spatio-temporal cluster analysis.

## 3.9. Appendix

Table 3.5. Number of treatments, and mean, median, standard deviation (SD), coefficient of variation (CV), minimum and maximum for the adult lice level one week after treatment, by neighbourhood.

| Neighbourhood | Number of treatments | Adult lice mean abundance | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Median | SD | CV | Minimum | Maximum |
| 2 | 11 | 2.0 | 1.5 | 1.8 | 0.9 | 0.2 | 6.2 |
| 3a | 42 | 16.2 | 5.8 | 25.2 | 1.6 | 0.2 | 86.7 |
| 3b | 10 | 6.2 | 6.1 | 2.2 | 0.4 | 2.2 | 8.7 |
| 6 | 22 | 2.4 | 1.8 | 2.1 | 0.9 | 0.2 | 8.2 |
| 8 | 21 | 5.2 | 3.7 | 5.2 | 1.0 | 0.2 | 20.4 |
| 9a | 65 | 3.4 | 3.0 | 2.3 | 0.7 | 0.2 | 11.0 |
| 9b | 10 | 5.3 | 5.2 | 2.7 | 0.5 | 1.3 | 8.7 |
| 9c | 11 | 3.0 | 1.9 | 2.1 | 0.7 | 0.4 | 6.2 |
| 10a | 24 | 4.7 | 4.7 | 2.6 | 0.6 | 0.3 | 9.4 |
| 10b | 27 | 6.4 | 5.3 | 5.1 | 0.8 | 0.5 | 20.9 |
| 11 | 14 | 6.2 | 3.5 | 6.3 | 1.0 | 1.0 | 22.7 |
| 12a | 8 | 3.0 | 2.5 | 2.4 | 0.8 | 0.1 | 6.4 |
| 12b | 1 | 0.2 | 0.2 | . | . | 0.2 | 0.2 |
| 16 | 40 | 5.9 | 2.2 | 11.6 | 2.0 | 0.0 | 66.4 |
| 17a | 10 | 2.9 | 1.3 | 3.1 | 1.1 | 0.1 | 8.3 |
| 17b | 15 | 1.1 | 0.9 | 0.7 | 0.6 | 0.2 | 2.8 |
| 18a | 114 | 12.6 | 2.9 | 34.4 | 2.7 | 0.0 | 282.0 |
| 18b | 7 | 9.4 | 7.7 | 7.2 | 0.8 | 0.3 | 19.5 |
| 18c | 15 | 5.2 | 3.3 | 3.8 | 0.7 | 1.4 | 15.0 |
| 18d | 48 | 8.0 | 5.3 | 9.8 | 1.2 | 1.3 | 53.4 |
| 18e | 11 | 4.7 | 4.9 | 1.9 | 0.4 | 0.6 | 7.6 |
| 19a | 12 | 5.4 | 6.5 | 2.9 | 0.5 | 0.5 | 8.7 |
| 19b | 39 | 5.3 | 4.8 | 3.0 | 0.6 | 2.0 | 15.9 |
| 20 | 96 | 12.0 | 5.8 | 19.3 | 1.6 | 0.4 | 92.0 |
| 21a | 6 | 3.5 | 3.7 | 1.3 | 0.4 | 1.5 | 5.0 |
| 21b | 15 | 4.8 | 3.9 | 2.3 | 0.5 | 1.4 | 8.8 |
| 21c | 28 | 5.0 | 5.1 | 2.2 | 0.4 | 0.8 | 8.7 |
| 22a | 13 | 8.0 | 6.9 | 8.2 | 1.0 | 0.5 | 28.0 |
| 22b | 23 | 5.0 | 4.2 | 5.4 | 1.1 | 0.9 | 26.9 |
| 22c | 1 | 2.8 | 2.8 | . | . | 2.8 | 2.8 |

Table 3.5. (continued).

| Neighbourhood | Number of treatments | Adult lice mean abundance | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Median | SD | CV | Minimum | Maximum |
| 22d | 21 | 3.8 | 3.9 | 2.5 | 0.7 | 0.0 | 9.6 |
| 23a | 27 | 6.1 | 5.1 | 3.5 | 0.6 | 0.6 | 17.4 |
| 23b | 18 | 5.7 | 4.7 | 3.4 | 0.6 | 1.6 | 15.3 |
| 23c | 17 | 4.7 | 4.8 | 2.5 | 0.5 | 0.2 | 10.1 |
| 24 | 64 | 7.5 | 4.5 | 12.8 | 1.7 | 0.7 | 91.0 |
| 26a | 7 | 5.2 | 6.5 | 3.0 | 0.6 | 1.3 | 8.7 |
| 26b | 3 | 1.6 | 1.7 | 0.2 | 0.1 | 1.4 | 1.8 |
| 27 | 8 | 3.6 | 3.5 | 3.2 | 0.9 | 0.3 | 8.6 |
| 28a | 43 | 4.8 | 4.7 | 2.9 | 0.6 | 0.2 | 12.1 |
| 28b | 1 | 0.0 | 0.0 | . | . | 0.0 | 0.0 |
| 30a | 16 | 4.0 | 4.6 | 2.5 | 0.6 | 0.2 | 8.2 |
| 30b | 8 | 4.4 | 4.3 | 2.1 | 0.5 | 1.9 | 7.8 |
| 31a | 22 | 2.5 | 2.2 | 2.1 | 0.8 | 0.1 | 8.3 |
| 33 | 1 | 0.4 | 0.4 | . | . | 0.4 | 0.4 |
| 34 | 75 | 5.1 | 4.1 | 3.7 | 0.7 | 0.1 | 19.4 |
| Total | 1,090 | 6.9 | 4.1 | 14.8 | 2.1 | 0.0 | 282.0 |

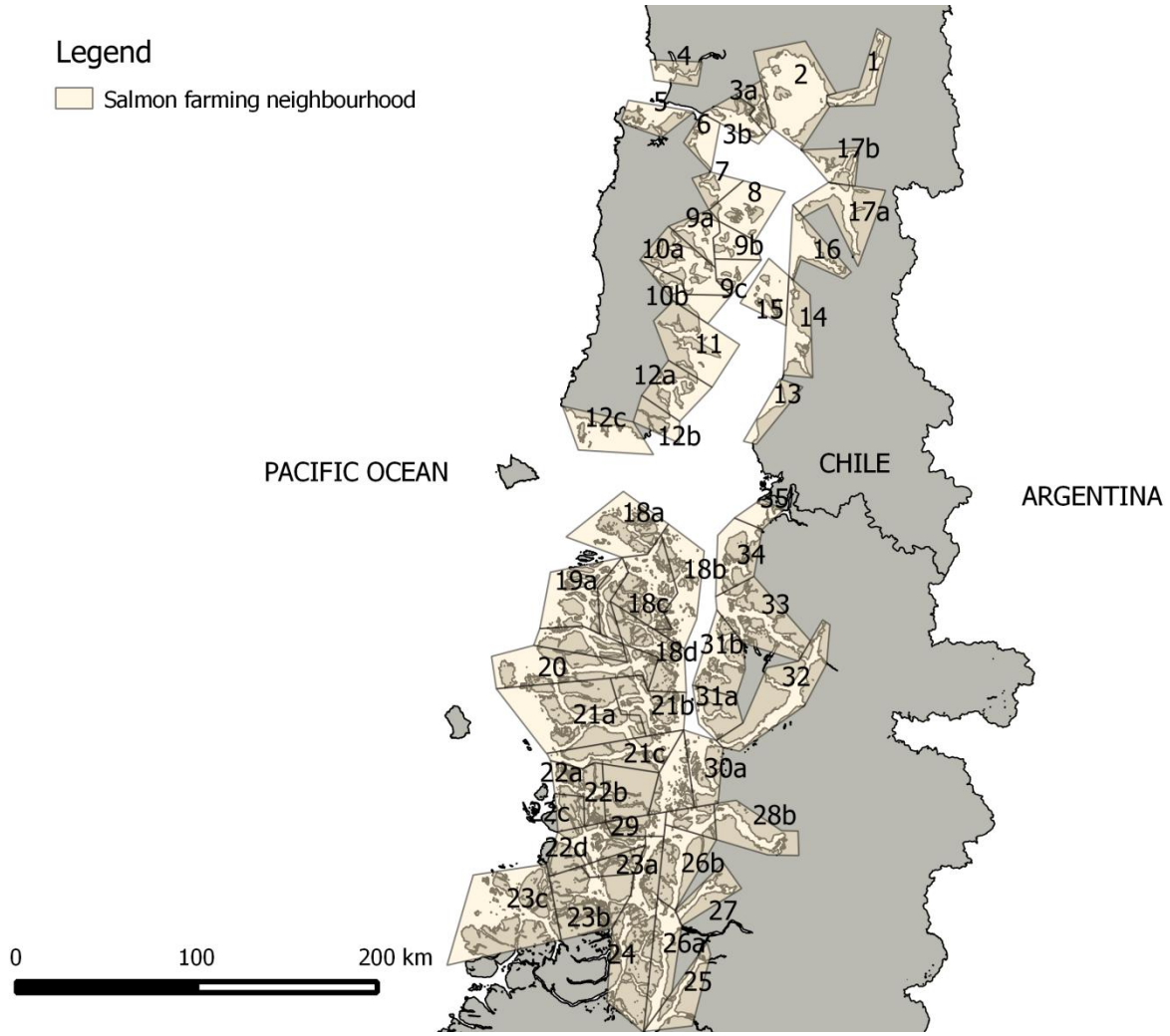


Figure 3.6. Study area and salmon farming neighbourhoods.

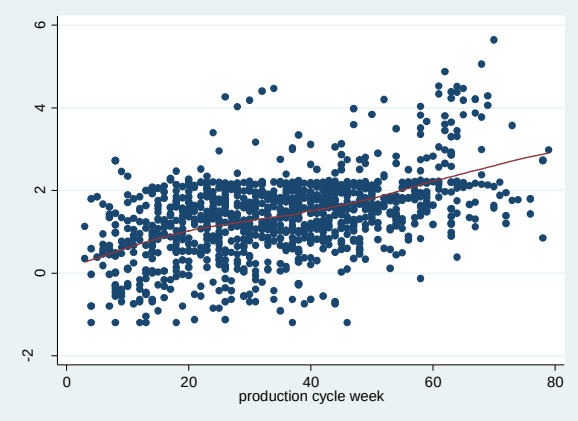


Figure 3.7a. Scatter plot for the log adult lice levels one week after a pyrethroid treatment over production cycle weeks.

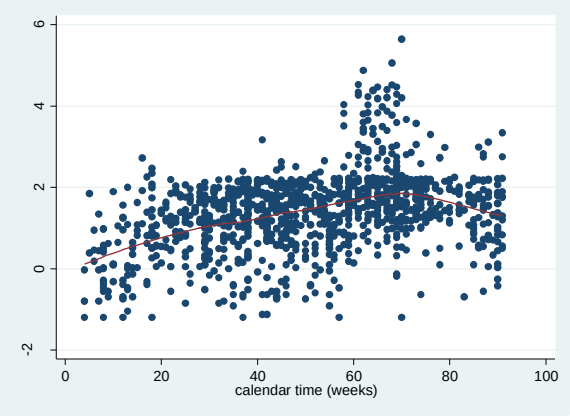


Figure 3.7b. Scatter plot for the log adult lice levels one week after a pyrethroid treatment over calendar time.

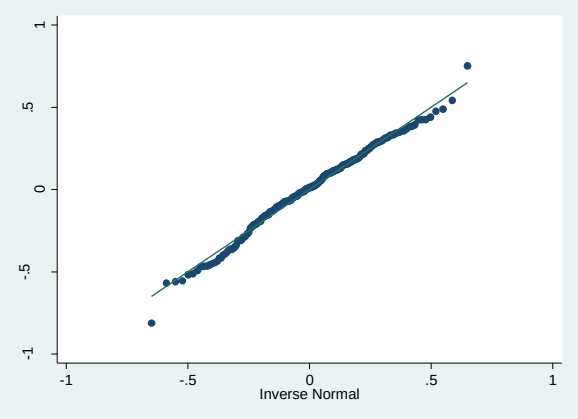


Figure 3.8a. Q-Q plot for farm effect predictions.

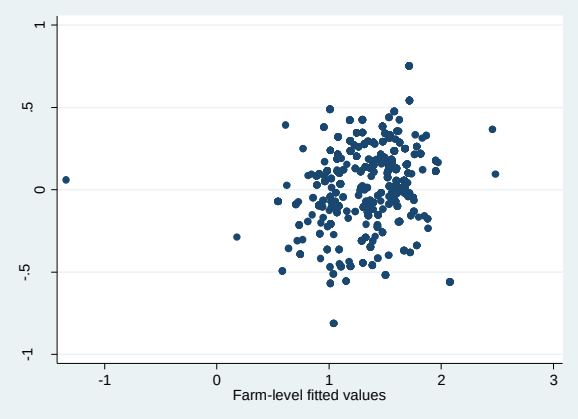


Figure 3.8b. Residual plot for farm effect predictions.

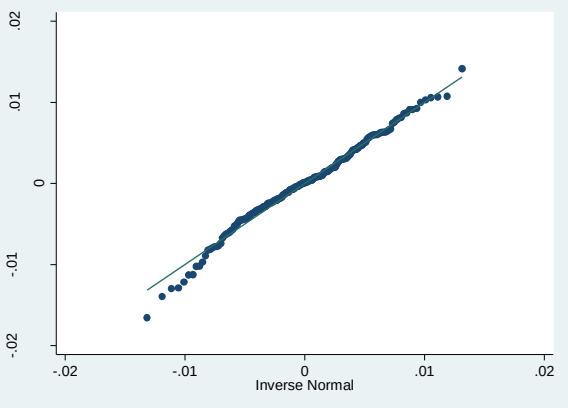


Figure 3.9a. Q-Q plot for production cycle week random coefficient (farm level).

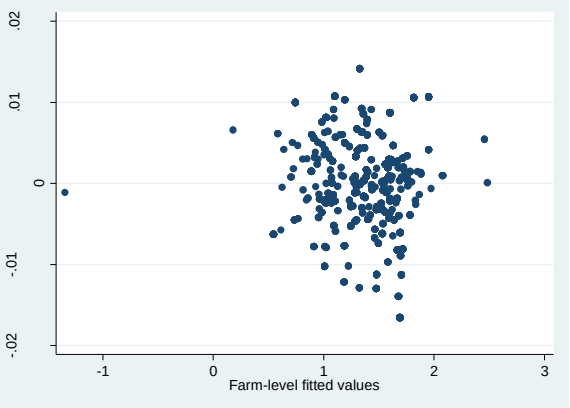


Figure 3.9b. Residual plot for cycle week random coefficient (farm level).

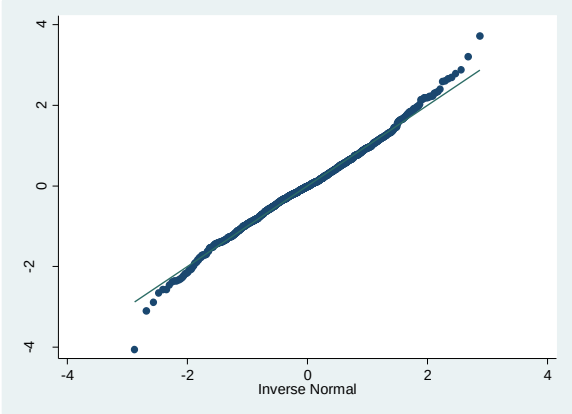


Figure 3.10a. Q-Q plot for standardized residuals.

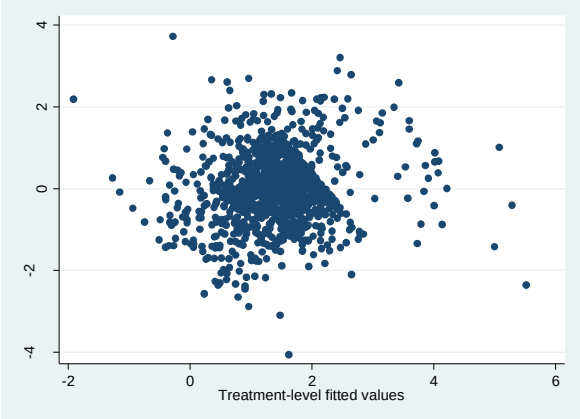


Figure 3.10b. Residual plot for treatment-level standardized residuals.

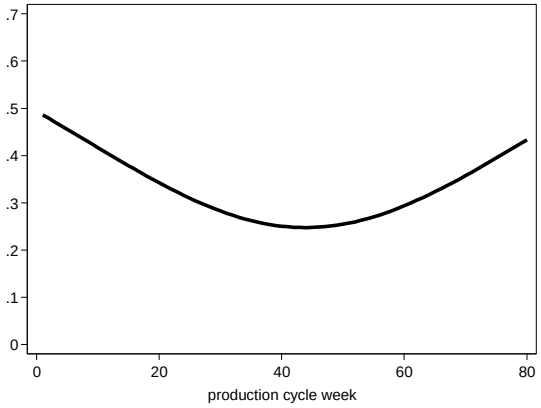


Figure 3.11. Farm level intra-class correlation coefficient (farm ICC) as function of the production cycle week.

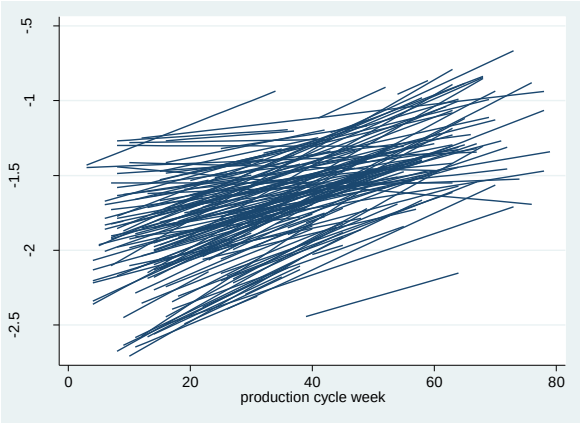


Figure 3.12. Predicted farm slopes for production cycle week.

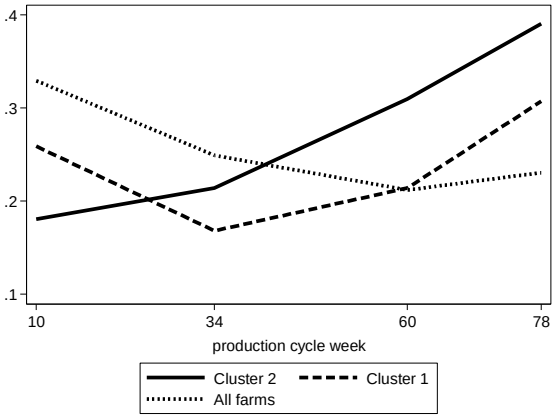


Figure 3.13. Standard deviation (SD) among farm predictions from full model within two spatial clusters (Table 3.3) and overall among all farms.

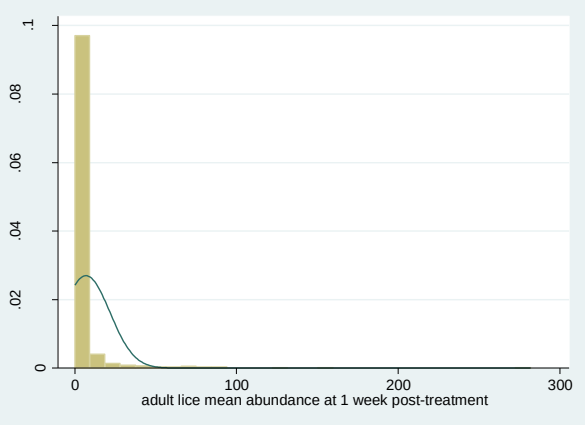


Figure 3.14a. Histogram for adult lice mean abundance at 1 week post-treatment (raw data).

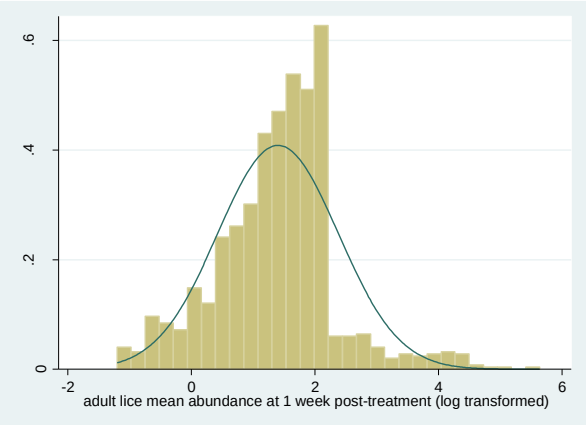


Figure 3.14b. Histogram for adult lice mean abundance at 1 week post-treatment (log transformed data).

# CHAPTER 4

# EVALUATING THE EFFECT OF SYNCHRONIZED SEA LICE TREATMENTS IN CHILE

## 4.1. Abstract

Sea lice is considered the most important ectoparasite that affects salt water farmed salmonids around the world. Its high economic impact has motivated efforts for its control. Immersion pharmacological treatments (i.e. baths) are the most used control strategy in several salmon-producing countries, including Chile. As a topical procedure, immersion treatments do not induce a long lasting effect; therefore, re-infestation from neighbouring farms may undermine their efficacy. Synchronization of treatments has been proposed as a strategy to improve immersion treatment performance. We used a repeated measures linear mixed effect model to evaluate treatment synchronization of neighbouring farms (within one week and 10 km window) on the adult lice mean abundance from weeks 2 to 8 post-treatment on rainbow trout and Atlantic salmon farms in Chile. We controlled for external and internal sources of lice before the synchronization, and also for environmental and fish related variables. Results indicate that treatment synchronization was significantly associated with lower adult lice levels at weeks 5 and 6 after treatment. This relationship appeared to be linear, suggesting that higher levels of synchronization may result in lower sea lice levels at these weeks. These findings suggest that synchronization can improve immersion treatment performance by keeping sea lice levels low for a longer period of time. Our results may be applicable to other regions of the world where immersion treatments are widely used.

## 4.2. Introduction

The sea louse is an ectoparasite that affects farmed and wild salmonids in the marine phase and is considered one of the main health challenges for the salmon industry worldwide (Costello, 2006; Burka et al., 2012). This parasite increases costs to salmon farms by inhibiting fish growth, causing extensive skin damage which makes the fish more susceptible to other pathogens, and reducing marketability (Costello, 2009).

Globally, the most used tool for sea lice control is anti-lice drugs; however, in recent years treatment failures have been reported in most salmon-producing regions (Sevatdal & Horsberg, 2003; Sevatdal et al., 2005; Bravo et al., 2008; Lees et al., 2008; Jones et al., 2012). This situation has motivated investigations of the performance of anti-lice treatments, revealing that one cause of treatment failure is the low sensitivity of sea lice to certain chemicals (Sevatdal & Horsberg 2003; Sevatdal et al., 2005; Bravo et al., 2008). More recently, research has focused on improving the drug administration methods; in particular, with immersion treatments (i.e. baths) (Corner et al., 2011), which involve complex procedures at the farm. Sea lice re-infestation from external sources is another factor that can impair treatment performance by increasing the post-treatment levels. Several studies have found external sources of lice are significantly associated with sea lice abundances at the farm level (Aldrin et al., 2013, Jansen et al., 2012, Kristoffersent et al., 2014). A recent study identified different sources of sea lice in Chile and determined that the infection pressure from neighbouring farms was greater than that coming from within the farm itself (Kristoffersen et al., 2013). The recent increase in use of immersion treatments, which do not provide protection for as long as oral treatments, has made re-infection from external sources a greater concern than ever. Consequently, if external sources of lice are ignored, the efforts undertaken within the farm might be seriously limited or undermined.

One strategy that has been implemented worldwide that helps in controlling external sources of sea lice is coordinated treatments (Rae, 1999; Jackson, 2011; Revie, 2011; Ritchie & Boxaspen, 2011; Saksida et al., 2011), which consist in the application of delousing treatments in all farms within a management zone, in a relative short period of time. The rationale behind this approach is to interrupt the sea lice life cycle at all farms at the same time; this should minimize the exchange of larvae among farms after the treatments and keep the sea lice levels low over time (Ritchie & Boxaspen, 2011).

Coordinated treatments can have different objectives. In Norway, Ireland, Scotland, and the western coast of Canada, coordinated treatments are performed at specific times of the year (once or twice a year) to

reduce sea lice levels on farms and transmission to wild fish (Rae, 1999; Jackson, 2011; Revie, 2011; Ritchie & Boxaspen, 2011; Saksida et al., 2011). These procedures have been referred to as strategic coordinated treatments, as they target specific lice stages at specific times of the year. In Norway, for example, the winter coordinated treatment is intended to eliminate overwintering female lice, which are responsible for the spring peak of juvenile lice when water temperatures rise, while the spring coordinated treatments are aimed at killing the newer cohort of lice (Ritchie & Boxaspen, 2011).

In Chile, in contrast, coordinated treatments are aimed of improving the treatment performance; to that end, treatment coordination is encouraged all year round by establishing coordinating windows of 7 days of duration every approximately 2 weeks for each of the eight macro-areas in the country (Sernapesca, 2012). Because treatments in Chile need to be carried out in a relative short period of time, the term "synchronized" is a better descriptor of the activity than "coordinated".

There are few published studies that have evaluated the effect of (strategic) coordinated treatments for controlling sea lice in farmed fish. One of them, carried out in Scotland between 1993 and 1996, found that strategic coordinated treatments performed in winter were associated with a reduction of both sea lice levels and treatment numbers, when comparing two consecutive production cycles, one with and one without coordinated treatments (Wadsworth, 1998). The univariable nature of this study and the low number of sites (n=5) make it difficult to extrapolate much from these findings. Another study in Scotland, involving a higher number of farms and a longer period of time, observed a relationship between the timing of treatments and the mobile counts (Revie et al., 2002), suggesting that concentration of treatments in time reduced sea lice abundance. In contrast, when these data were analyzed with a multivariable approach, the sea lice abundance on farms that participated in the strategic treatments was no different from those that did not (Revie et al., 2003); however, it was later suggested that this result may have been seriously impacted by confounding (Revie, 2011).

There are no published studies that have evaluated the effect of treatment synchronization on sea lice levels over time. The Chilean context, which involves monthly voluntary synchronized treatments, weekly sea lice monitoring, and a large number of fish farms, offers a unique opportunity to evaluate treatment synchronization at the farm level, while controlling for external sources of lice and factors that affect the sea lice abundance at the farm itself. The objective of this research was to assess the duration of the effect of synchronized treatments on sea lice levels while controlling for the initial treatment effect on farms and other potential confounders.

## 4.3. Methods

### 4.3.1. Study Location

Our study was conducted in Los Lagos and Aysén regions (41°28' to 46°18') in southern Chile. This area consists in a 500 x 150 km system of small channels, fjords and islets, which contains approximately 90% of salmon farming activity in the country. Atlantic salmon and rainbow trout are the most commonly grown species reaching approximately 70% of the active farms in 2012-2013, while Coho (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*) represent the rest.

### 4.3.2. Data and study period

Data originated from the Chilean salmon farming association's (SalmonChile) sea lice monitoring program. This association collects and manages information on approximately 90% of salmon farms located in the study area. Each participating farm reports *Caligus rogercresseyi* counts for juvenile (chalimus I to IV), mobile adults (including non-gravid females), and gravid female stages on 10 fish from each of four pens (40 fish in total) on a weekly basis. Weekly sea lice assessments are performed following the protocols described in the Official Caligidosis Surveillance and Control Program (Sernapesca, 2012). From these count data a mean abundance for each life stage is calculated for each farm. Information about delousing treatments is also reported to the SalmonChile's database, specifying the product used and the starting/finishing dates of the procedure. Environmental data such as water

temperature and salinity, and certain production information are also collected on a weekly basis. We restricted the data in our study from January 2012 to September 2013 because of a change in the method of reporting treatments in the database which occurred in late 2011.

### 4.3.3. Selection of treatment events

Treatment events were obtained from the full list of delousing treatments reported through SalmonChile's sea lice monitoring program during the study time period. Each treatment event was followed in time until a new delousing treatment occurred or to a maximum of eight weeks post treatment. Our study was restricted to treatments performed with topical drugs, because these treatments are more prone to fail due to re-infestation shortly after treatment, as they do not have residual effects on fish as do oral treatments. Topical drugs reported during the study period were azamethiphos and synthetic pyrethroids, the latter including deltamethrin and cypermethrin. Treatments performed with more than one drug were not included in this study. We further discarded treatment events with only one week post-treatment, as we were not interested in modeling the drop of sea lice levels after the treatment, because another study has recently addressed this issue (Chapter 2). In addition, we included only treatment procedures lasting at most one week in order to avoid exceptionally long procedures. Finally, we excluded treatment events carried out in farms with no neighbouring farms within 10 km seaway distance due to the fact that we used this distance as the limit for the synchronization window (see section 4.3.5). Delousing treatments were carried out by the farmers based on their own criteria, following the manufacturers' directions.

### 4.3.4. Study design and outcome variable

The study design was structured as a retrospective cohort study. Our outcome of interest was the adult *C. rogercresseyi* mean lice abundance at the farm level after a delousing treatment, starting from the second week and up to the eighth week after the procedure. We did not included the first week post-treatment in the outcome because we were not interested in modeling the drop of sea lice levels right after the treatment, given that another study recently addressed this issue on *C. rogercresseyi* (Chapter 2). We

defined this follow-up period based on the *C. rogercresseyi* life cycle (González & Carvajal, 2003; Bravo, 2010), which suggests that the potential effect exerted by lice from neighbouring farms could be observed up to 7 weeks after the procedure. We chose adult *C. rogercresseyi* as the outcome, because this life stage appeared to be more sensitive to synthetic pyrethroids than the juvenile stages (Chapter 2). Adult *C. rogercresseyi* included male, non-gravid and gravid female lice. The mean adult lice abundance (Rózsa et al., 2000) was based on the total 40-fish sample reported every week through the sea lice monitoring program.

#### 4.3.5. Treatment synchronization variable

In order to build this variable we first set a spatio-temporal window for each of the treatment events that met the inclusion criteria described in section 4.3.3. We used this window to identify the neighbouring farms that applied an immersion treatment simultaneously with the farm of interest. Considering that delousing treatments highly concentrated in time are expected to have a better outcome (Jackson, 2011; Ritchie & Boxaspen, 2011) and that an average farm (approximately 20 cages) usually treat all cages within a week (R. Ibarra, pers. comm.), we set the synchronization window duration at one week, which, for the sake of simplicity, corresponded to the calendar week in which the treatment procedure started at the farm of interest. Calendar weeks were denoted with numbers according to the ISO 8601 date and time standard (ISO, 2004), weeks starting on Monday. The first week of the year is the week that contains that year's first Thursday; the highest week number in a year is either 52 or 53. For this study, we considered all weeks in 2012 (i.e. 52) and the first 40 weeks in 2013.

With regard to the geographical range of the window, we took into consideration a recent study by Kristoffersen et al. (2013) which observed a dose-response relationship between the sea lice abundance at the farm level and the distance to neighbouring farms. Because the effect should be greater with closer neighbours, we decided to evaluate the coordination within a 10 km seaway distance in order to determine the presence of a treatment synchronization effect.

The next step was to quantify the treatment synchronization. In areas with intensive salmon farming activity, like southern Chile, transmission of sea lice between farms is also strong (Jackson, 2011; Ritchie & Boxaspen, 2011). In the absence of a delousing treatment or other control measure, the larval flow towards neighbouring farms should be continuous as the lice life cycle progresses. When effective delousing treatments are applied, the larval production should be significantly reduced through the elimination of adult lice, particularly gravid females. Findings from Kristoffersen et al. (2013) suggest the number and proximity of neighbouring farms are strong drivers of external sea lice infection pressure. This means the potential reduction in the larval flow should be proportional to the number of farms with lice that treat and inversely proportional to the distance between these farms and the farm of interest. Consequently, one of the ways we quantified the treatment synchronization was the number of farms that reported immersion treatments within the synchronizing window. In practice, we considered a neighbouring farm as synchronized if at least one day of its own treatment procedure coincided within the synchronization window.

We did not consider in-feed treatments in our synchronization measures because these drugs are used in a relative low proportion in comparison with topical drugs (R. Ibarra, pers. comm.), and because the Chilean regulation only encourages synchronization of treatments performed with topical drugs.

The treatment synchronization intensity (TSI_1) was determined as follows: for each immersion treatment (*g*) included in our study, we weighted neighbouring farms (*j*) within 10 km seaway distance of the treatment farm (*i*) that reported an immersion treatment in the same week, where each weight was determined from the seaway distance $d_{ij}$, and a Gaussian kernel density ($w_{10k}(d_{i,j})$), as used by Kristoffersen et al. (2013). Seaway distances were calculated using the *gdistance* package in R v.3.0.1 (www.r-project.org). Geographic coordinates for each farm were provided by SalmonChile. In formulae, the treatment synchronization intensity for a given treatment event ($TSI\_1_g$) was calculated as follows:

$$\text{TSI\_1}_g = \sum_{j \in R(g)} w_{10k}(d_{i,j}) \quad , i = farm(g), \qquad \text{(Eq. 1)}$$

where *R(g)* is the synchronizing window of 1 week of duration and 10 km seaway distance, from treatment *(g)*.

The Gaussian kernel weights ($w_{10k}(d_{i,j})$) for each neighbouring farm (*j*) relative to the farm of interest (*i*) were calculated as follows:

$$w_{10k}(d_{i,j}) = \frac{1}{\sqrt{2\pi}\tau} e^{-\frac{1}{2\tau^2}{d_{ij}}^2}$$

where $d_{ij}$ is the seaway distance between the farm of interest (*i*) and the neighbouring farm (*j*). The bandwidth choice was 5.25 km.

TSI_1 was a measure that did not consider the total number of neighbouring farms around the farm of interest, which, in turn, represents the maximum potential of treatment synchronization in the area. In order to correct for this effect, we also included a variable representing the maximum synchronization potential (MSP). MSP was calculated as the sum of kernel weighted distances between neighbouring farms with sea lice and the farm of interest within a 10 km seaway distance, similar to the way as TSI_1 was calculated, but including all the neighbouring farms irrespective of whether they treated or not.

TSI_1 mostly represents treatment synchronization by chance; however, because the legislation in force at the time of this study encouraged the synchronization of treatments within a 7-to-10-day window once a month (see section 1.3.7.2), it is possible that TSI_1 represents actual treatment synchronization at some extent. That would happen in the case that our synchronization window matches the Government's.

#### 4.3.5.1. Alternatives measures for treatment synchronization

In addition to TSI_1, we created two other variables that also expressed treatment synchronization. The alternative treatment synchronization variables were: the number of farms that did not report immersion treatment during the synchronization window and had any sea lice (TSI_2), and the proportion of farms that reported immersion treatment during the synchronization window (TSI_3). We chose one of these three variables for representing treatment synchronization in the final model. We based our selection on model fit, using the Akaike Information Criteria (AIC), and on other criteria such as parsimony and practical interpretability.

TSI_2 was calculated in the same way as TSI_1 (see Eq. 1), but summing over the neighbouring farms that did not report immersion treatments. TSI_3 was computed by dividing the number of neighbouring farms that reported immersion treatments (TSI_1) by the total number of neighbouring farms (MSP), both terms calculated as the sum of kernel weights based on the distance between each neighbouring farm and the farm of interest within a 10 km seaway distance area (see Eq. 1).

The effect of neighbouring farms (all of them, treated or not) around the farm of interest was accounted for in TSI_3 by including MSP as a denominator. In the case of TSI_2, a variable representing the total number of neighbouring farms was not needed as it focuses on farms that will continue contributing to the external infectious pressure (i.e. non-treated farms), while it assumes that treated neighbouring farms do not.

In order to explore the possibility that the effect of treatment synchronization might be affected by the total number of farms in the area, we tested the significance of a three-way interaction including MSP, TSI_1 and weeks.

### 4.3.6. Selection of other predictors

Selection of predictors for the model building process was based on a causal diagram (see Figure 4.1) depicting our *a priori* knowledge about the factors that impact the adult lice abundance between 2 and 8 weeks after treatment.

#### 4.3.6.1. Other off-farm predictors

Based on Kristoffersen et al. (2013) we hypothesized that the infection pressure exerted by neighbouring farms in the surrounding 30 km before carrying out the treatment synchronization might affect the adult lice levels during the follow-up period given that free swimming larval stages are not affected by bath treatments on farms. Because information about sea lice larvae intensity around each farm of interest was not available in the Chilean context, we accounted for the external infectious pressure by including the reproductive potential of neighbouring farms within 30 km seaway distance from the farm of interest. This variable was expressed as the sum of gravid female mean abundances ($GF_{j,t(g)-1}$) at neighbouring farms ($j$) one week before the treatment ($g$) and within 30 km seaway distance of the treatment farm ($i$), each of those also weighted by seaway distance and a Gaussian kernel density ($w_{30k}(d_{i,j})$). The neighbouring farm's reproductive potential ($NRP_g$) was therefore calculated as follows:

$$\mathrm{NRP}_g = \sum_{j \in A_i} \mathrm{GF}_{j,t(g)-1} w_{30k}(d_{i,j}) \quad , \quad i = farm(g), \qquad \text{(Eq. 2)}$$

where $A_i$ is the area included within the 30 km seaway distance from the treatment farm ($i$).

The Gaussian kernel weights ($w_{30k}(d_{i,j})$) for each neighbouring farm were calculated as for TSI_1 (see section 4.3.5). The bandwidth choice is this case was 15.25 km.

We anticipated that the role of NRP and MSP is important in our system, and possibly these two variables may modify the effect of the synchronization variable. For that reason we tested interactions of NRP and MSP with TSI_1 and week in the model building process. In addition, we explored the autocorrelation of both NRP and MSP over time. To do that, we built a linear mixed model with NRP or MSP as the outcome, including a farm random effect and a first order autoregressive structure for residuals (AR1).

#### 4.3.6.2. On-farm predictors

**Internal source of lice**

The adult lice level before the treatment is another important variable to control for, because it is responsible for producing larval stages that were not affected by the on-farm treatment. According to the *C. rogercresseyi* life cycle (Bravo, 2010), these individuals could develop into adult lice as early as the third week after treatment if these larval stages attached to fish shortly after treatment. For these reasons, we tested the mean gravid female lice one week before the immersion treatment as a measure of the internal source of lice to adjust for this effect.

**Treatment-related variables**

Another group of variables with a potential impact on the adult lice level during the follow-up period were those related to the treatment performed on the farm of interest. One of these factors was the drug used in the treatment procedure at the farm of interest. Although it is known that topical drugs such as those used in this study do not have a residual effect on fish, and that by including the sea lice levels one week after treatment (see later in this section) we accounted for this effect, we considered it valuable to include this variable due to its possible association with area level features; for example, areas with high sea lice levels may have preferred a particular drug. Topical drugs reported by farms during the study period included the synthetic pyrethroids, deltamethrin and cypermethrin, and the organophosphate azamethiphos.

In addition, it is known that the time taken to treat all cages on a farm impacts the post-treatment sea lice levels. When treatments take a long period of time to complete, the first treated pens can be re-infected by lice from pens that have not yet been treated (Costello, 2006). Longer periods for treatments are common when the administration method is immersion treatment (i.e. baths) because the procedure is complex and time consuming. Consequently, we included the time in days that it took to treat the entire farm.

The potential effect of previous delousing treatments during four weeks before the treatment in evaluation was assessed by including a 5-level categorical variable with the following categories: no treatment (reference level), azamethiphos, pyrethroid, emamectin benzoate, and mixed treatments.

**Environmental and fish-related variables**

Water temperature and water salinity are environmental variables well known for impacting the *C. rogercresseyi* abundance as these variables affect survival, fertility, and development of lice (González & Carvajal, 2003; Zagmutt-Vergara et al., 2005; Bravo et al., 2008; Bravo, 2010; Yatabe et al., 2011); therefore, we included them in the model building process.

Biological aspects of the host as well as the husbandry conditions also impact the development of sea lice (Costello, 2006; Torrissen et al., 2013). In particular, observational studies have found statistically significant associations of fish weight, total number of fish, fish biomass, and stocking density, with *C. rogercresseyi* abundance (Zagmutt-Vergara et al., 2005; Yatabe et al., 2011; Kristoffersen et al., 2013; Chapter 2). In our study, this group of variables may influence the adult lice levels as extraneous variables during the follow-up period so we included these variables measured one week prior to the respective sea lice abundance value.

**4.3.6.3. Time**

A theoretical study on *Lepeophteirus salmonis* population dynamics over a period of less than 100 days

suggested that sea lice increase exponentially over time (Krkošek et al., 2010), unless effective control measures are taken. *C. rogercresseyi* would be expected to exhibit a similar behaviour. We were interested in evaluating the effect of synchronized treatments on sea lice over time and we recognized that the effect likely differed as time elapsed after the treatment. In fact, several predictors in our model likely had different effects at different times post treatment (for example, treatment synchronization, internal and external sources of lice), so we included time in our model and its interactions with those predictors.

### 4.3.7. Statistical models

The log of adult lice mean abundance at week *t* after treatment *g* was modeled using a linear mixed effects model with neighbourhood, farm, and treatment event random effects, and a correlation structure to account for the repeated measures (weeks) within each treatment event. Originally, the model was built adding an offset of 0.01 to the mean abundance of adult lice before the log transformation; however, in order to explore whether a better model fit may be achieved, we tried other offset values in the range between 0.01 and 1.5, following a Box-Cox procedure described by Venables & Ripley (1999) and a visual assessment of Q-Q plots of standardized residuals at the week level. The model equation was expressed as:

$$\ln(Y_{gt} + 0.01) = X_{gt}\beta + u_g + u_{farm(g)} + u_{neighb(g)} + \varepsilon_{gt} \quad \text{(Eq. 3)}$$

where $Y_{gt}$ is the adult lice mean abundance, $X_{gt}$ is the vector for fixed effects, $\beta$ is the corresponding coefficient vector, while $u_g$, $u_{farm(g)}$, and $u_{neighb(g)}$ are random effects for treatment event, farm and neighbourhood, respectively, all assumed to be independent and normally distributed, with mean zero and corresponding variances. Errors ($\varepsilon_{gt}$) were assumed to be correlated due to repeated observations in time and, consequently, this equation component was modeled with a suitable correlation structure allowing for the expected decay in correlation while increasing the time steps.

### 4.3.8. Interpretation of coefficients

Coefficients for variables not interacting with weeks in the final model were back-transformed into the original sea lice scale by exponentiating the base of the natural logarithm (*e*) to the corresponding (relative) coefficient value. In the case of variables interacting with weeks in the final model, we estimated coefficients for the variable of interest for each of the six weeks in the follow-up period. For doing so, we specified values for each of the independent variables in the final model, and predicted the effect of one unit increase of the predictor of interest in the log sea lice level. In order to simplify the interpretation of coefficients of predictors measured on the log scale, for example, the gravid female lice level one week before the treatment, we expressed the adjusted coefficients as the change in the adult lice level (i.e. our outcome in the original scale) relative to a two-fold increase of the predictor (i.e., $e^{\beta * \ln(2)}$).

### 4.3.9. Model building process and model validation

We log transformed (ln) the weekly averaged adult sea lice abundance to meet the normality and homoscedasticity assumptions of parametric tests based on a Box-Cox analysis. The model was built using the stepwise backward elimination approach, starting from a maximum model which included all the predictors described above. Variables with the highest *p*-values (Wald test) were removed from the model one at a time until all predictors were below the significance threshold ($p \leq 0.05$), unless the removal of the variable induced a substantial change in the coefficients of the other predictors (> 20%). It was assumed that these were confounders and so these were maintained in the model. When two explanatory variables were highly correlated ($|\rho| > 0.7$), only one of the predictors was retained in the model. This choice between them was based mostly on known biological effect.

Time was included as a categorical variable (week) with week 2 as the reference level. We included interaction terms between week and predictors plausible to have a time-varying effect. These predictors were: TSI_1, TSI_2, TSI_3, MSP, NRP, gravid female mean abundance 1 week before treatment, and adult and juvenile mean abundance 1 week after treatment. In case the interaction term were not

significant (Wald test $p<0.05$), we included the predictor as a single term (i.e. no interaction). If the single term was not significant, we definitively removed the predictor from the final model. In any case, our first choice was to include these predictors interacting with time, unless the predictor as a single term yielded a better fit, based on the Akaike Information Criteria (AIC). In cases the model fit was similar with and without the interaction with weeks ($\Delta AIC \leq 2$), we chose the predictor interacting with time for the final model. The reason for preferring more complex models is because we know the effect of certain predictors would be time-dependent, and we wanted to represent that in the final model.

The model was fitted using maximum likelihood (ML) estimation. Correlation between error terms was evaluated by exploring different correlation structures, such as first order autoregressive (AR(1)), Toeplitz, and unstructured with equal and unequal variances. We chose the best fitting correlation structure based on parameter significance, model parsimony, and whether the estimated $\rho$ (rho) had an expected decay between time steps. Model comparisons involving hierarchical and correlation structure were based on the likelihood ratio test and AIC. The assumption of linearity between continuous predictors and the outcome was inspected by running mean and locally weighted smoothing curves (lowess) between the standardized residuals and each continuous predictor retained in the final model. The homoscedasticity assumption for each random effect was inspected by plotting standardized residuals versus predicted values, while the normality assumption was assessed with Q-Q plots using the standardized residuals and predicted best linear unbiased predictors (BLUPs) for farm and neighbourhood random effects. The impact of extreme observations, defined by having standardized residuals numerically > 3, was evaluated by removing the observations from the model one at a time to assess their influence on the coefficients. Statistical analyses were performed with Stata IC 13 (StataCorp LP).

#### 4.3.10. Model predictions

We used the final model to predict adult lice levels during the follow-up period under different scenarios. The treatment synchronization variable was assessed by comparing the scenarios of null and high

synchronization intensity. In all cases, we predicted for the Aysén region and for pyrethroid treatments, while the rest of the predictors were set at their mean values.

#### 4.3.11. Diagrammatic interpretation of the associations observed in our final model

In order to better understand the effect of factors interacting with time, we provided a series of path diagrams to represent the causal relationships among predictors, where the strength and significance of the association are graphically depicted.

## 4.4. Results

### 4.4.1. Descriptive analysis

Out of 1,811 immersion treatments reported in the Intesal's Sea Lice Monitoring Program during the study period, 706 (39%) fulfilled our inclusion criteria. These treatments were performed on 227 farms located in 46 neighbourhoods across two fish farming regions in Chile. Four-hundred and eighty-one (481) of our treatments were performed on farms located in the Aysén region, while 225 were on farms in Los Lagos region. Six-hundred and four (604) sea lice treatments were carried out on Atlantic salmon and 102 on rainbow trout. During the study period the farms treated, on average, 6 times per production cycle, with 23% of farms treating 10 or more times. In total, 2,278 weekly sea lice measurements were included in our analysis. Fish included in the study weighed, on average, 2.56 kg, though weights ranged from 0.16 to 9.41 kg. Water temperature ranged between 7.4 and 16.7° C, with a mean of 10.9° C.

When the adult lice mean abundance was averaged at different levels of the predictors included in our analysis (raw data), distinct patterns were observed (Table 4.1). For example, the adult lice mean abundance appeared to increase until week 5 post-treatment. Regarding the external infection pressure we observed that, in general, as the number of neighbouring farms (only with Atlantic salmon or rainbow trout) increased, the mean sea lice level increased as well. A similar trend was observed with overall sea lice numbers for the study period with the number of neighbouring farms that reported an immersion

treatment. The effect of water temperature on our outcome exhibited a pattern characterized by higher levels of adult lice between 10 and 12° C, and lower levels at temperatures outside this range. There were other variables that presented consistent trends across different levels of the predictors; for example, stocking density, and sea lice levels one week before and one week after treatment. In these cases, as the level of the predictor increased, so did the adult lice level. For the choice of drug used in the treatment, the mean lice level on a farm did not appear to differ between the two drugs tested. Finally, before adjusting for other factors the sea lice levels were on average higher for Atlantic salmon than rainbow trout (Table 4.1).

### 4.4.2. Multivariable analysis

#### 4.4.2.1. Selection of the treatment synchronization variable

We built several models containing different combinations of one of the three treatment synchronization variables (TSI_1, TSI_2, or TSI_3) and other related variables such as NRP and MSP. The model containing TSI_1, and NRP and MSP exhibited the lowest AIC value (Table 4.2). In general, models containing TSI_1 exhibited the lower AICs, while models with TSI_2 or TSI_3 showed much greater AIC values. Models that included TSI_2 showed better fit than models with TSI_3 (Table 4.2).

Based on our criteria for selecting one of the measures for treatment synchronization (see methods), we chose the model containing TSI_1, and NRP and MSP (Model #1 in Table 4.2). This model showed that as TSI_1 increased (i.e. a greater number of neighbouring farms treated the same week as the farm of interest), the adult lice levels at the farm of interest were lower. Although the interaction of TSI_1 with time was borderline significant ($p$=0.067) (see Table 4.3), the magnitude and significance of the coefficients for the interaction terms suggest that synchronization impacted the adult lice levels at weeks 5 and 6 after treatment ($p$=0.009 and $p$=0.027, respectively). The effect of synchronization at week 7 was similar in magnitude to that at week 6; however, it was borderline significant ($p$=0.076). As the value of TSI_1 ranged between 0 and 0.25 units, we chose to interpret the coefficient for a change in 0.20 TSI_1

units (rather than one TSI_1 unit), which represents a high synchronization scenario. For example, 0.2 TSI_1 units could be achieved if a farm had four neighbouring farms at a distance of 4.8 km that treated. Under the high synchronization scenario (TSI_1=0.2), the predicted adult lice levels at weeks 5 and 6 post-treatment were 44% and 56% lower than the null synchronization scenario (TSI_1=0.00) (see Figure 4.2), after controlling for the other predictors in the model.

The number of susceptible farms in the synchronization area (MSP) was associated with significantly greater adult lice levels during the follow-up period ($p$=0.045). A model including this variable as interacting with weeks showed the effect of MSP did not significantly vary across the follow-up period ($p$=0.212) (Model #3 in Table 4.2); therefore, MSP was kept in the final model without interacting with weeks to control for the number of potential farms that could have treated for sea lice in the 10 km area around our farm of interest. The 3-way interaction term between TSI_1, weeks and MSP was not significant ($p$=0.264).

#### 4.4.2.2. Other off-farm predictors

The inclusion of NRP in the final model was associated with a decrease of 21.1 AIC units (see models #9 and #1 in Table 4.2). We found there was a positive association between NRP one week before the treatment synchronization and the adult lice levels during the follow-up period ($p$<0.001). The NRP effect did not significantly vary across weeks in the follow-up period ($p$=0.417) (see model #4 in Table 4.2). One extra unit of NRP (which could represent, for example, a farm that has four neighbouring farms at 2.5 km, each with an average of 10 gravid female lice per fish) impacted the farm of interest by 20% more adult lice during the follow-up period. When we tested for a potential modifying effect of NRP on the TSI_1 effect, we found the 3-way interaction term between TSI_1, week, and NRP was not significant ($p$=0.314).

Our final model indicated that the level of adult lice during the follow-up period was also influenced by the region where the farm was located. If the farm was in the Aysén region, the adult lice abundance was, on average, 18% higher ($p$<0.001) (Table 4.3).

#### 4.4.2.3. On-farm predictors

Our model indicated that the abundance of both adult and juvenile lice one week after treatment had a positive impact on the adult lice level during the follow-up period. In both cases, the effect significantly varied across weeks, although adult lice showed a stronger variation in time ($p$<0.001), in comparison to juvenile lice ($p$=0.033). The effect of adults was significant at each of the seven weeks in the follow-up period, while juvenile's effect was significant at the first two. The estimates indicate that the effect of adult lice was greater at week 2 after treatment (40% more adult lice per two-fold adult lice at week 1 after treatment) and then it decreased to 14% more lice at week 8. The estimates indicate the effect of juvenile at weeks 2 and 3 after treatment to have been 14% and 18% more adult lice per doubling the juvenile lice at week 1 after treatment, respectively.

Our final model shows the level of gravid females one week before the treatment was significantly associated to the adult lice level and it varied across the follow-up period ($p$<0.001), in particular from week 4 onwards (Table 4.3). The estimates indicate that doubling gravid female the week before the treatment, the post treatment adult lice abundance will increase between 15% and 31%.

The drug used to treat lice on the farm of interest, was significantly associated with our outcome ($p$=0.016). We found that when pyrethroids were used, the adult lice level was 24% higher in comparison with azamethiphos. We found the treatment duration (i.e. time taken for treating all cages in the farm) did not show a significant impact on the adult lice level during the follow-up period ($p$=0.856), therefore, it was removed from the final model. Previous delousing treatments in the same farm during 4 weeks before the treatment in evaluation did not have a significant impact ($p$=0.161) on the adult lice levels.

Among the four fish-related production variables we tested, stocking density showed a significant association with the adult lice level during the follow-up period ($p$=0.002). Its effect was positive and appeared to be linear within the range of stocking density values in our dataset. For each additional kg of fish per cubic meter at the farm, the mean adult lice abundance increased, on average, by 1.7%. Fish weight, total number of fish and fish biomass were not significant in our final model ($p$=0.456, 0.072, 0.107, respectively)

Water temperature was the only environmental variable that remained in the final model. It showed a positive and significant association with our outcome ($p$<0.001). Per each additional degree Celsius on water, the adult lice level increased, on average, 4.8%. Water salinity was not significant in our final model ($p$=0.312), thus it was dropped.

#### 4.4.2.4. Variance components and residual structure

The variance estimate of the farm random effect was not significantly different from zero ($p$=0.302), indicating that no significant clustering of adult lice levels was observed across farms, after controlling for the other predictors in our model. However, due to the inherent hierarchical nature of the data we maintained this effect in the final model. The inclusion of the neighbourhood random effect did not have any impact in the model fit (the variance estimate was equal to zero); therefore, it was removed from the final model.

The unstructured arrangement with unequal variances had the best fit to the data so this was chosen (Table 4.4). Following this arrangement for residuals, the model showed an increasing trend for the error variances with time, with a small drop at week 8 (Table 4.3). In general, the covariance between pairs of weeks showed a slow decay as the time step increased.

#### 4.4.2.5. Model fit

Standardized residuals for the lowest level of our model (weeks) showed some departure from normality (Figure 4.4b). This was mostly driven by a group of observations with extreme negative residuals. The percentage of observations with standardized residuals greater than numerically 3 was only 1.7%. The variance of standardized residuals appeared to be constant across fitted values (i.e. homoscedastic) (Figure 4.4a). After fitting a model without these extreme outliers, the coefficient of the synchronization variable (TSI_1) at week 7, and the coefficients of juvenile post-treatment level at weeks 4 and 5 became significant. BLUPs for farm random effects showed no major departure from normality and approximately constant variance (Figures 4.5a and 4.5b). All continuous predictors, including those that were interacted with weeks, exhibited a linear relationship with the outcome.

Models built with other offsets for the outcome (e.g. 0.3, 0.5, 1.0, 1.3 and 1.5) improved the left tail of the distribution of standardized residuals (relative to normality), but worsened the right tail (data not shown), therefore, we kept 0.01 as the offset for the final model. The major trends in the estimates for the synchronization effect were not affected by the choice of the offset value.

## 4.5. Discussion

We found that a treatment synchronization procedure within a 1-week and 10-km seaway distance window was associated with a decrease in abundance of adult sea lice levels at weeks 5 to 7 after treatment.

### 4.5.1. Treatment synchronization effect

We evaluated the effect of synchronized sea lice treatments on the abundance of adult *C. rogercresseyi* on farms 2 to 8 weeks post treatment, controlling for the amount of gravid females in the surrounding area and other factors affecting post-treatment sea lice levels. Among the three different treatment synchronization variables we evaluated, we chose TSI_1 because of its better fit. Our study suggests that

the more synchronized the treatments were, within 10 km seaway distance and a 1 week period, the lower the levels of adult lice were at weeks 5 to 7 post-treatment (even if the impact at week 7 was only borderline significant). We consider this a plausible effect, i.e. that the synchronization of treatments on neighbouring farms resulted in a reduction in the number of gravid females and, consequently, the number of infective copepodids in the area. Our chosen measure of synchronization combined the number of neighbours that treated and their distance from the farm of interest. This mean that high levels of synchronization could have been achieved either when a greater number of farms treated at the same time, and/or when these treated farms were closer to the farm of interest. Our findings are in agreement with other researchers who have found strong evidence for the role of neighbouring farms as external sources of sea lice in Chile (Kristoffersen et al., 2013), Norway (Jansen et al., 2012; Aldrin et al., 2013), and Scotland (Adams et al., 2012; Murray & Hall, 2014).

The pattern of the predicted lice abundance observed over time (Figure 4.2) may be explained by both the life stages that are targeted by bath treatments and the effect of treatment synchronization. The increase in adult lice in the early weeks after treatment suggests that, despite the treatment, the louse life cycle was still active. The study presented in Chapter 2 found that pyrethroids are less effective against juvenile stages of *C. rogercresseyi*. Therefore, the increase in adult sea lice during weeks 2 to 4 after treatment may be the consequence of surviving juvenile lice that evolved into adult stages.

The drop in adult lice levels at weeks 5, 6, and 7 suggests the source of juvenile lice at the farm of interest was somehow substantially reduced before that time. This may be due to the treatment synchronization effect because, hypothetically, this procedure reduces the larval flow from neighbouring farms and, therefore, the availability of juvenile lice at the farm of interest. Any constraint in the larval availability would result in a reduction in the adult lice counts around 32 days later (4.5 weeks), because, according to Bravo (2010), this is the time that *C. rogercresseyi* eggs need to become adult lice at 11° C. This explanation is in agreement with our results.

Our model suggests that the treatment synchronization effect lasted at least 2 weeks. This probably reflects the fact that treatments in neighbouring farms eliminated many of the gravid females in the area, but new gravid females emerged and resumed the production of larval stages shortly after treatment due to surviving adult and juvenile lice that eventually mature. According to Bravo (2010), chalimus III and IV need between 7 and 10 days to evolve into gravid females at 11° C, in agreement with our findings. By week 8, the effect of synchronization seems to have disappeared (Figure 4.2), which suggests that between 2 to 3 weeks after the synchronized treatments the source of larvae (gravid females) has recovered.

It is important to note that TSI_1 coefficients during weeks 5, 6, and 7 post-treatment were strongly negative (in comparison with the rest of the weeks), and increased numerically over time, which suggests that during that 3-week period the treatment synchronization effect escalated. However, the significance level decreased at the same time, and was totally non-significant by week 8 (Table 4.3 and Figure 4.3). Based on this, we could not determine whether the diminishing significance over time was due to a biological phenomenon or the progressive reduction in the sample size during the follow-up period (Table 4.1).

Figure 4.2 shows that the predicted adult lice level under the null synchronization scenario (TSI_1=0.0) did not show an exponential trend, which is the theoretical tendency of sea lice development dynamics according to Krkošek et al. (2010). Furthermore, the predicted pattern decreased at week 8 post-treatment. This may be an artifact of the study design, given that farms that contributed more weeks of data to the study were those that did not require new treatments, possibly because their sea lice levels were not high enough to trigger a treatment. This situation may have occurred later in the follow-up period (week $\geq 6$), as it is more likely to occur as the time period without treatments increases. The decline in the mean adult lice abundance from week 6 onwards observed in the descriptive analysis (Table 4.1) supports this explanation; thus, the effect of treatment synchronization may have been underestimated in the last few

weeks of the follow-up period. Another explanation for the decrease of adult lice level observed at week 8 is the fact that, according to regulations, farms were forced to treat whenever the sea lice level was equal to or greater than 6 adults per fish (Sernapesca, 2012). In such conditions, we dropped the treatment week and following of that particular farm from the analysis, according to our inclusion criteria (see section 4.3.4); leaving in the study farms with more than 7 weeks without treatments, which generally are farms with lower sea lice levels.

### 4.5.2. Off-farm variables

We confirmed that the neighbouring farm infectious pressure, expressed in our case as NRP, had a positive association with the sea lice abundance at the farm level, which suggests a direct link between the area's gravid females and our outcome. The influence of the infectious pressure on sea lice abundance has been studied by other researchers for both *C. rogercresseyi* and *L. salmonis*, with results similar to ours, although infectious pressure has been expressed differently. For example, Kristoffersen et al. (2013) worked with an estimate of the absolute number of gravid females within 30 km in their study, which aimed to explain juvenile *C. rogercresseyi* abundance. In contrast, Jansen et al. (2012), who modeled mobile *L. salmonis*, expressed it as the total fish biomass within 40 km.

The final model also included MSP, which represents the amount of active fish farms in the area before the treatment synchronization. Both NRP and MSP were built in a similar manner; however, NRP covers a wider area (30 km seaway distance) and includes gravid female lice on neighbouring farms. That may explain why NRP seemed to have a much more important role in the system compared to MSP. This relative importance can be quantified by the change in the model fit when models with and without the predictor (i.e. NRP) were compared (Table 4.2). For example, when NRP was added to model #12 AIC dropped more than 25 points, while in the equivalent situation for MSP, AIC decreased only 6 points.

We found that one of the reasons for the limited impact of MSP was the presence of NRP in the final model. When we evaluated the impact of MSP in a model without NRP (see Model #9 in Table 4.2), we observed the significance level of the MSP coefficient increased and the drop in the AIC value (models with and without MSP) was greater than when NRP was present (see Models #1 and #2 in Table 4.2). Despite this apparent relationship between NRP and MSP, correlation between these two predictors was relatively low (Pearson correlation r = 0.19). This supports the notion that NRP and MSP are actually representing different aspects in our system. While NRP is representing quite accurately the amount of gravid females, and consequently the volume of larvae in an area, MSP considers only the presence and proximity of neighbouring farms in a much shorter range (i.e. 10 km). We think NRP represents the sea lice dynamics in space in a much better way than MSP.

Neither NRP nor MSP seemed to exert any obvious confounding effect on TSI_1 or any other predictor in the final model. This suggests the effect of these two predictors on our outcome is direct (i.e. exposure-independent variable), meaning it is not mediated through any other predictor. This fact highlights the need for a measure of the production status in the area (number of farms) in order to properly evaluate treatment synchronization.

We also explored the possibility that the level of NRP or MSP might modify the effect of TSI_1. We studied this through a three-way interaction between TSI_1, weeks, and NRP or MSP. Neither of these interactions was significant, suggesting that the relationship between synchronization and sea lice levels was independent of the level of farms around the site (MSP) and the level of gravid lice in the neighbourhood (NRP).

Further, our results showed that interactions between NRP or MSP with time (weeks) were also not significant. This suggests that the impacts of NRP or MSP at their particular spatial scales on sea lice were roughly of the same magnitude at each week in the follow-up period. This situation challenges the

biology of sea lice which suggests the effect of NRP or MSP (assuming their effect is mediated by the larval flow) should be highly variable in time. These effects should be observable only from the week 4 or 5 after treatment because larvae need approximately this period of time for evolving into adult lice (Bravo, 2010). We hypothesize that this situation may be the consequence of a high correlation of NRP values across consecutive weeks, meaning that the single NRP value we have for each treatment is actually a surrogate variable for NRP values some weeks before the procedure. High autocorrelation values observed for NRP and MSP support this hypothesis.

### 4.5.3. Alternative measures of treatment synchronization

Compared to TSI_1, the alternative measures of treatment synchronization TSI_2 and TSI_3 resulted in poorer model fits (Table 4.2). Thus TSI_1 better represented the sea lice dynamics over time in the study area. We can only speculate about the reasons behind these differences. One obvious difference between TSI_1 and TSI_2, for example, is that the latter focuses on the farms that did not treat during the synchronizing window, while the former focused on farms that treated. Despite both variables being built from the same data, the value zero for TSI_1 was present twice as frequently as it was for TSI_2 (see Figures 4.6a and 4.6b). As zero is the base of comparison for estimating the effect, this difference may affect the coefficient estimation.

The substantial difference between models including TSI_1+MSP and TSI_3, for example models #11 and #13 in Table 4.2, is that in the first case the number of farms that treated and the total number of farms in the area were included as two different variables, while in the second case these variables were included as a proportion. The consistently better fit of models with TSI_1 (+MSP) compared to models containing TSI_3 (Table 4.2) may be due to the fact that proportions are constrained between 0 and 1 while TSI_1 and MSP are not. It is also possible that MSP on its own captured other effects at the area level.

### 4.5.4. TSI_1 as a measure of treatment synchronization variable

It is important to remember that TSI_1 was not recorded as a variable itself, but that it was created from previously recorded information. Variables constructed from large databases, like the one used in this study, might exhibit spurious associations with others due to chance, or may represent different features than those for which they were intended. However, there are key arguments to suggest that TSI_1 appropriately represents treatment synchronization, and the association observed with our outcome has a consistent biological explanation.

The strength of these results is that they fit well with what we expected in terms of the biology of the parasite and the Chilean salmon farming setting. Two key findings that exemplify this are the timing and the duration of the TSI_1 effect. The timing of the TSI_1 effect matches well the *C. rogercresseyi* life cycle as the effect of treatment synchronization is mediated by larval stages, and larvae need at least 4 weeks to become adult lice (Bravo, 2010). Similarly, the duration of the TSI_1 effect also agrees with the louse biology because the time it takes for a population of sea lice to resume the larvae production after a successful delousing treatment (targeted to adult lice) is approximately 15 days.

The way that TSI_1 was built is another argument in its favour as an appropriate treatment synchronization variable. TSI_1 is an index as it combines a number of predictor variables that are related into a single predictor that represents some overall level of a factor (Dohoo et al., 2009, page 370). Indices can be constructed in a subjective or an objective way, depending on how the researcher links mathematically and weights the individual factors that build the index. In our case, TSI_1 was built in an objective manner, as these aspects were informed by biologic evidence. First, TSI_1 is an additive index as it captures the expected dose-response relationship between the treatment intensity in the area and our outcome by summing the neighbouring farms that treated. Second, we weighted each treated farm's contribution to the index by its proximity to the farm of interest. Furthermore, farm weights were calculated using a Gaussian kernel density which assumes the relationship between the impact of a

neighbouring farm and its distance is not linear, but it increases when distance between farms is shorter. This refinement was intended to reflect the fact that larval stages remain at a greater concentration close to the farm of origin (Torrissen et al., 2013).

It is also helpful to speculate on what other factors or conditions could cause the drop observed in the adult lice level at weeks 5, 6, and 7 associated with our measure of treatment synchronization. A situation that may produce a similar result to our study is a quick harvest of fish from neighbouring farms, which would result in a decreased larval flow to the farm of interest. However, in this hypothetical case it would be very unlikely that the level of adult lice recover (like in our study) because there would not be fish to maintain the larval production in the area. In addition, this possibility assumes that several quick harvests will occur frequently along the production cycle of neighbouring farms, but this is not the way that fish farms operate. Another situation that may be associated to a reduction in adult lice level is delousing treatments; however, this does not seem likely as we deliberately designed our study to avoid extra treatments during the follow-up period (see section 4.3.3).

### 4.5.5. Practical implications

Our findings suggest that treatment synchronization may reduce the treatment frequency at the industry level. Between January 2012 and September 2013, farms in Chile administered on average 6 treatments per production cycle with 23% of sites treating 10 or more times. The Chilean legislation requires farms to treat if they have fish with 6 or more adult lice in the proximity of a farm that has 9 or more adult lice per fish (i.e. also called a high dissemination farm) (Sernapesca, 2012). Predictions from our model indicate that when delousing treatments are performed individually (i.e. without synchronizing with the neighbours) the adult lice level on an average farm will exceed this threshold around the 5$^{th}$ week after treatment, for other predictors set at average values. By contrast, when all farms with susceptible fish (i.e. rainbow trout and Atlantic salmon) within the 10-km seaway distance joined the treatment synchronization, the treatment threshold, on average, would be not exceeded until after the 7$^{th}$ week post

treatment. This 2 to 3-week delay in the increase of parasite level could mean delaying the next treatment on a farm, or increasing the time between treatments and, therefore, performing fewer treatments during the production cycle. Fewer treatments would have environmental and economic benefits. Reducing the use of chemotherapeutants may also reduce the rate of development of resistance (Denholm et al., 2002).

### 4.5.6. Other predictors

We found that gravid female lice recorded at one week before the treatment was positively associated with the adult lice level during the follow-up period, suggesting the gravid female population on a farm may have played a role as a source of lice. These results are consistent with Kristoffersen et al. (2013), who studied the effect of gravid female lice levels two weeks prior, on juvenile *C. rogercresseyi* levels. In our case, we also learned that the gravid female level impacted the adult lice level from week 4 onwards, which is consistent with the life cycle of *C. rogercresseyi* that needs around 5 weeks for an egg to develop into an adult at 11° C (Bravo, 2010).

Our model indicated that the resulting juvenile and adult lice levels, one week after the treatment, had a positive impact on our outcome. This reflects the fact that lice that survived the treatment continue evolving into the adult stage. The adult level one week post-treatment impacted significantly our outcome at each week in the follow-up period, but at a decreasing rate over time (see Figure 4.3). This is probably the result of the progressive death of these adults counted one week after treatment. The juvenile group was positively associated with adult sea lice only at weeks 2 and 3, showing a decreasing effect in time (see Figure 4.3), suggesting that most of the juvenile individuals recorded one week post-treatment evolved into adults after 2 weeks, which is consistent with the *C. rogercresseyi* life cycle.

Consistent with other experimental and observational research that have examined the effect of water temperature on *C. rogercresseyi* (González & Carvajal, 2003; Zagmutt-Vergara et al., 2005) and *L. salmonis* (Johnson & Albright, 1991; Jansen et al., 2012) development, we found a significant and

positive effect of water temperature on adult lice levels during the follow-up period. In addition, we found that stocking density also had a positive effect on our outcome. This result was in agreement with other studies on *C. rogercresseyi*, such as Yatabe et al. (2011). A possible reason for the significant association of stocking density with our outcome is that larvae in water have a greater chance to land on a suitable host when fish densities are high.

The choice of drug at the farm of interest impacted the adult lice level during the follow-up period. Treatments performed with pyrethroids seemed to have a higher adult lice mean abundance than azamethiphos even after correcting for the initial levels of lice after treatment; however, as these drugs do not have a long lasting effect (i.e. they are topical), we believe the drug choice at the farm of interest is representing other effects in our system. An explanation for this situation is that pyrethroids are more commonly used in areas with higher levels of sea lice; therefore, the larval exchange is greater in these areas, which ends up with higher sea lice levels at the farm of interest.

Unlike other observational studies regarding *C. rogercresseyi* (Zagmutt-Vergara et al., 2005; Yatabe et al. 2011; Kristoffersen et al., 2013; Chapter 2), our study showed that water salinity did not have a significant influence on the adult lice level on farms, after adjusting for covariates. We found its significance was affected by allowing unequal variances in the residual correlation structure. A similar situation occurred with other week-level variables in our model, such as average fish weight. The reason for such an effect is that ignoring unequal variances (i.e. heteroscedasticity) may produce incorrect standard errors, and consequently, inaccurate *p*-values (Dohoo et al., 2009).

### 4.5.7. Variance decomposition

The model effects that represent the hierarchical structure of the data showed, first, that region had a significant effect on the adult lice levels, which suggests there are other variables that also impact the adult lice level (not included in our model) that vary between regions. One variable that meets these

conditions is the level of compliance of the immersion treatment procedure (according to regulations and manufacturer's guidelines). It is known that the logistics of applying treatments are more difficult in the Aysén region (R. Ibarra, pers. comm.), which may have reduced the effect of the treatments on farms in that area compared the to Los Lagos area.

After trying several correlation structures for the residuals, we chose the unstructured arrangement with unequal variances as it produced the lowest AIC, despite the relatively large number of parameters, compared to simpler structures (see Table 4.4). From this process of comparison among different correlation structures we found that a substantial improvement in the model fit was achieved when we included an unequal variance residual structure, meaning that the variance of the adult lice level changed over time. In fact, the variance estimates showed a clear, increasing trend in time, which makes biological sense because, as weeks pass, the factors that affect the increasing rate of sea lice are different in each situation, producing greater dispersion of data. Based on our final model's covariate estimates, we found that the correlation decay in time was slower than the one estimated with the AR1 correlation structure.

### 4.5.8. Model fit

Our major concern regarding the model fit was the normality assumptions of the week-level residuals (Figure 4.4b). In general, other offsets explored for the outcome transformation improved the adjustment in one extreme of the distribution but it worsened in the other, therefore, no substantial gain was achieved, in comparison with the final model (offset=0.01). In addition, models built with different offsets yielded similar results as the final model. For these reasons, we kept 0.01 as the offset for the final model. As a consequence of that decision, we have to admit that the ability of the final model for predicting low sea lice levels is worse than for predicting medium or high sea lice levels.

Most of the extreme residuals that seemed to drive the departure of normality in the final model were negative. After checking these extreme negative residuals we found that most of them (>80%) were

observations with observed levels of lice equal to zero (or very close), and because most of them were at week 3 or greater during the follow-up period, the predicted value of lice was relatively high, producing a large negative residual. Drops like that in the sea lice levels, with no management intervention, are considered unusual assuming that, theoretically, sea lice increase exponentially over time (Krkošek et al., 2010). We attributed this situation to non-reported delousing treatments during the follow-up period. We retained these data points but they may have biased our findings towards the null.

#### 4.5.9. Bias and limitations

The definition of the temporal dimension of our synchronization window was a complex task in which we tried to capture most of the variability associated with the different timing of treatments (i.e. treatment start and finish) performed at both the farm of interest and neighbouring farms, in a simple and straightforward way (see section 4.3.5). However, there are some extreme situations in which our treatment synchronization measure could fail in representing the actual timing of treatments across farms. One of these situations is when the treatment at the farm of interest started at the end of a calendar week (e.g. Sunday) (and therefore the synchronization window was set at that calendar week), and another procedure at a neighbouring farm finished at the beginning of the same calendar week (e.g. Monday). In that case, our synchronization measure assumed these two treatments occurred simultaneously, but, in reality, it was not the case. Consequently, the treatment synchronization measure (e.g. TSI_1) for that particular treatment event would be greater than it should be. This may have biased the effect of the treatment synchronization variable at some extent, but we are confident these extreme cases occurred at low frequency in our dataset. Other treatment synchronization measures based on more refined criteria should be studied in future research.

As mentioned earlier, missing data was present in our dataset, associated to farms that retreated during the follow-up period (i.e. before week 8 post-treatment). Because farms that treat more frequently are those with problems for controlling lice, it is likely that missing values are associated with higher sea lice

levels. However, it is possible that the data is missing at random (i.e. the probability of missing data is completely driven by a factor already included in our analysis, such as juvenile and adults 1 week after treatment), and therefore valid inferences may have been made even if the missing mechanism was not explicitly considered in the analysis (Joseph et al., 2004).

Our treatment synchronization variable (TSI_1) did not consider the efficacy of the treatment carried out by the neighbouring farms. As we included only whether the farm reported a delousing treatment or not, we assumed an equal treatment efficacy in all cases, which is incorrect, given that we could observe a wide range of treatment efficacies on farms. Future research should consider this fact in the evaluation of area-level treatments.

We did not consider in-feed treatments in the treatment synchronization variable (TSI_1) building (see section 4.3.5). That may have underestimated the treatment synchronization impact because we are not accounting for potential reductions in the larval flow from neighbouring farms using these types of drugs. However, due to reported resistance and low treatment efficacy of EB (Bravo et al., 2008), and because the contribution of in-feed treatments in our dataset was relatively low (approximately 11% of all delousing treatments), we think the inclusion of synchronized in-feed treatments would not have a great impact on our findings.

Because we did not have information regarding sea lice levels in wild fish; therefore, we could not control for the role that wild fish may have on spread infection among farms or in treatment performance.

Another situation that may have affected our results is when our synchronizing window matched the Government's window and farms actively synchronized treatments within it. Because in these cases synchronization is performed at the neighbourhood level (~40 km wide), the actual number of farms joining the coordinated procedure would be greater than the farms we included in our 1week-10km

window. This situation may produce an extra synchronization effect which we are not considering in our analysis that could have potentially biased our results.

We evaluated the effect of treatment synchronization in a 1-week and 10-km synchronization window on farms with any level of lice (lice abundance >0). It is difficult to convince producers to treat their fish if there are very few lice on their animals and no clinical effect from sea lice infections. It may be that farms with low levels of lice do not have a large impact on the synchronization effect in an area, in which case it may not be necessary to include them in the area-wide treatment synchronization. This would reduce the use of chemotherapuetants that are not absolutely required. Future research should consider assessing of other sea lice thresholds (e.g. lice >3) to determine the synchronization settings that have the greatest impact on the sea lice levels over time. Future studies should also evaluate time to a specific sea lice level to determine whether synchronized treatments reduces the time it takes a farm to achieve a specified treatment threshold.

### 4.6. Conclusions

Anti-parasitic treatment failures can be due to low sensitivity of sea lice to drugs, inadequate drug administration procedure, and/or re-infestation from external sources of sea lice, such as infected neighbouring farms. Our study provides, for the first time, evidence that the synchronization of delousing treatments within 10 km seaway distance may improve treatment effect.

In particular, our results suggest that the synchronization of treatments in a 1-week and 10-km seaway distance window reduced the adult lice levels on farms at the week 5 and 6 post-treatment, delaying the rise of lice for at least 2 weeks. We also observed a linear relationship between the intensity of synchronization and the reduction of sea lice burdens, suggesting that full synchronization of farms within the synchronizing window produced better results than partial and no synchronization. It is important to note that despite synchronization the adult lice levels steadily increased up to 4 weeks after treatment.

This may be due to the fact that juvenile are not killed effectively and free swimming larval stages are not affected by the immersion treatments.

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## 4.8. Tables and figures

Table 4.1. Descriptive statistics for the adult lice mean abundance at different levels of selected variables included in the model building process.

| Variables | Levels | Mean | Median | 90% range | n |
|---|---|---|---|---|---|
| Week post-treatment (follow-up period) | 2 | 7.70 | 4.28 | 0.55 – 19.05 | 704 |
| | 3 | 8.76 | 5.45 | 0.93 – 23.80 | 557 |
| | 4 | 9.74 | 5.85 | 0.75 – 25.65 | 368 |
| | 5 | 9.86 | 5.04 | 0.70 – 38.40 | 256 |
| | 6 | 8.30 | 5.13 | 0.48 – 24.40 | 171 |
| | 7 | 9.19 | 5.00 | 0.58 – 24.60 | 127 |
| | 8 | 6.87 | 4.55 | 0.33 – 20.50 | 95 |
| Number of neighbouring farms within a 10 km seaway distance (excluding farms with Coho salmon) [1] | 1 | 7.88 | 5.00 | 0.50 – 28.18 | 653 |
| | 2 | 6.17 | 4.65 | 0.75 – 17.75 | 613 |
| | 3 | 9.29 | 5.16 | 0.69 – 21.75 | 420 |
| | 4+ | 11.51 | 5.08 | 0.68 – 44.33 | 592 |
| Number of neighbouring farms that reported immersion treatment within 10 km seaway distance the same week as in the treatment farm [1] | 0 | 7.73 | 5.00 | 0.68 – 23.03 | 857 |
| | 1 | 6.74 | 4.85 | 0.70 – 18.50 | 890 |
| | 2 | 12.18 | 5.88 | 1.30 – 24.60 | 236 |
| | 3+ | 14.06 | 4.58 | 0.50 – 91.23 | 295 |
| Drug [1] | azamethiphos | 8.09 | 4.60 | 0.33 – 28.70 | 165 |
| | pyrethroids | 8.67 | 4.98 | 0.70 – 23.20 | 2113 |
| Duration (days) of the treatment procedure in the farm (limited to 7 days) | ≤ 2 | 8.73 | 4.25 | 0.53 – 24.73 | 971 |
| | 3 – 4 | 7.70 | 5.34 | 0.75 – 21.95 | 986 |
| | 4 – 7 | 11.14 | 5.93 | 1.25 – 28.80 | 321 |
| Water temperature (°C) | < 10 | 5.95 | 4.98 | 0.45 – 12.95 | 663 |
| | 10 – 12 | 10.55 | 5.00 | 0.75 – 40.00 | 1104 |
| | > 12 | 7.94 | 4.80 | 0.68 – 24.73 | 511 |
| Water salinity (ppt) | < 31 | 9.37 | 5.85 | 0.58 – 28.90 | 888 |
| | 31 – 32 | 8.22 | 4.36 | 0.75 – 19.50 | 1012 |
| | >32 | 7.95 | 5.01 | 0.63 – 20.50 | 378 |
| Species [1] | Atlantic salmon | 8.96 | 5.03 | 0.70 – 26.83 | 1928 |
| | Rainbow trout | 6.80 | 4.54 | 0.50 – 14.38 | 350 |
| Stocking density (kg/m$^3$) | < 5 | 5.54 | 3.88 | 0.45 – 12.95 | 746 |
| | 5 – 10 | 8.83 | 5.10 | 0.78 – 22.23 | 1008 |
| | > 10 | 12.63 | 6.34 | 1.63 – 51.35 | 524 |
| Gravid female lice mean abundance one week before treatment | 0 – 3 | 4.45 | 3.58 | 0.38 – 10.53 | 1138 |
| | 3 – 6 | 8.51 | 6.16 | 1.33 – 23.23 | 870 |
| | > 6 | 26.62 | 8.51 | 1.80 – 112.38 | 270 |
| Adult lice mean abundance one week after treatment | 0 – 3 | 4.45 | 3.50 | 0.45 – 10.48 | 1425 |
| | 3 – 6 | 9.01 | 6.73 | 2.05 – 24.55 | 562 |
| | > 6 | 28.33 | 9.60 | 3.95 – 109.60 | 291 |
| Juvenile lice mean abundance one week after treatment | 0 – 3 | 5.13 | 3.85 | 0.50 – 12.63 | 1553 |
| | 3 – 6 | 8.59 | 6.64 | 1.85 – 21.23 | 404 |
| | > 6 | 25.59 | 8.50 | 2.15 – 107.40 | 321 |

[1] adult lice mean abundances calculated across time series (2 to 8 weeks after treatment).

Table 4.2. Difference in the AIC, ΔAIC, between the top ranked model in Table 2 (AIC=4646.9) and models using different alternatives for representing treatment synchronization, and other related predictors.

| Model [a] | Treatment synchronization variable [b] | Other related variable(s) [c] | AIC | ΔAIC | Treatment synchronization variable *p*-value [e] |
|---|---|---|---|---|---|
| 1 | TSI_1 [d] | NRP + MSP | 4646.9 | 0 | 0.067 |
| 2 | TSI_1 [d] | NRP | 4648.9 | 1.9 | 0.068 |
| 3 | TSI_1 [d] | NRP + MSP [d] | 4650.7 | 3.8 | 0.061 |
| 4 | TSI_1 [d] | NRP [d] + MSP | 4655.1 | 8.2 | 0.024 |
| 5 | TSI_1 [d] | NRP [d] | 4656.9 | 10.0 | 0.025 |
| 6 | TSI_1 [d] | NRP [d] + MSP [d] | 4657.3 | 10.4 | 0.038 |
| 7 | TSI_2 [d] | NRP [d] | 4657.6 | 10.7 | 0.234 |
| 8 | TSI_3 [d] | NRP [d] | 4667.8 | 20.9 | 0.830 |
| 9 | TSI_1 [d] | MSP | 4668.0 | 21.1 | 0.078 |
| 10 | TSI_2 [d] | | 4670.8 | 23.9 | 0.207 |
| 11 | TSI_1 [d] | MSP [d] | 4672.7 | 25.8 | 0.064 |
| 12 | TSI_1 [d] | | 4674.2 | 27.3 | 0.081 |
| 13 | TSI_3 [d] | | 4682.8 | 35.9 | 0.882 |

[a] All models were run on the same subset of observations (n=2,278).
[b] Treatment synchronization variable is based on farms that did report immersion treatments (TSI_1), on farms that did not (TSI_2), and the proportion of farms that did reported immersion treatments, upon the total number of farms in the synchronizing window (TSI_3).
[c] Other related variables are: the reproductive potential of neighboring farms within 30 km (NRP), and the maximum synchronization potential (MSP).
[d] Predictor included as interacting with weeks.
[e] Test for interaction between the treatment synchronization variable and weeks.

Table 4.3. Coefficient estimates, standard errors and *p*-values for explanatory variables in the final model for the log adult *C. rogercresseyi* mean abundance from week 2 to week 8 post-treatment, on Atlantic salmon and rainbow trout farms in Chile (n=2,278).

| Variable name | Estimate | Standard error | *p*-value | 95% confidence interval | |
|---|---|---|---|---|---|
| **Fixed effects parameters** | | | | | |
| Intercept | -0.305 | 0.180 | 0.091 | -0.657 | 0.481 |
| Week after treatment (week 2 as reference) | | | **<0.001** | | |
| 3 | 0.561 | 0.045 | **<0.001** | 0.473 | 0.648 |
| 4 | 0.413 | 0.075 | **<0.001** | 0.265 | 0.560 |
| 5 | 0.661 | 0.085 | **<0.001** | 0.496 | 0.827 |
| 6 | 0.736 | 0.126 | **<0.001** | 0.489 | 0.984 |
| 7 | 0.791 | 0.183 | **<0.001** | 0.433 | 1.150 |
| 8 | 0.618 | 0.166 | **<0.001** | 0.292 | 0.945 |
| Treatment synchronization intensity (1w10k) | -0.208 | 0.525 | 0.691 | -1.237 | 0.802 |
| Treatment synchronization intensity (1w10k) * week | | | 0.067 | | |
| 3 | -0.331 | 0.460 | 0.473 | -1.223 | 0.572 |
| 4 | -0.341 | 0.856 | 0.691 | -2.019 | 1.337 |
| 5 | -2.651 | 1.020 | **0.009** | -4.650 | -1.652 |
| 6 | -3.850 | 1.740 | **0.027** | -7.260 | -0.439 |
| 7 | -3.997 | 2.253 | 0.076 | -8.413 | 0.419 |
| 8 | -0.436 | 1.989 | 0.827 | -4.335 | 3.463 |
| Neighbourhood reproductive potential pre-treatment (30 km) | 0.186 | 0.036 | **<0.001** | 0.115 | 0.257 |
| Maximum synchronization potential (10 km) | 0.835 | 0.416 | **0.045** | 0.020 | 1.650 |
| Log of gravid female mean abundance one week before treatment | 0.017 | 0.030 | 0.571 | -0.042 | 0.077 |
| Log of gravid female mean abundance one week before treatment * week | | | **<0.001** | | |
| 3 | 0.008 | 0.031 | 0.803 | -0.052 | 0.067 |
| 4 | 0.320 | 0.050 | **<0.001** | 0.223 | 0.419 |
| 5 | 0.255 | 0.054 | **<0.001** | 0.149 | 0.361 |
| 6 | 0.186 | 0.079 | **0.018** | 0.032 | 0.341 |
| 7 | 0.268 | 0.129 | **0.037** | 0.016 | 0.521 |
| 8 | 0.368 | 0.121 | **0.002** | 0.131 | 0.604 |
| Log of adult mean abundance one week after treatment | 0.488 | 0.027 | **<0.001** | 0.435 | 0.542 |
| Log of adult mean abundance one week after treatment * week | | | **<0.001** | | |
| 3 | -0.257 | 0.027 | **<0.001** | -0.311 | -0.204 |
| 4 | -0.309 | 0.047 | **<0.001** | -0.402 | -0.216 |
| 5 | -0.323 | 0.053 | **<0.001** | -0.427 | -0.220 |
| 6 | -0.319 | 0.077 | **<0.001** | -0.471 | -0.168 |
| 7 | -0.323 | 0.094 | **0.001** | -0.508 | -0.139 |
| 8 | -0.304 | 0.084 | **<0.001** | -0.468 | -0.140 |

Table 4.3. (continued)

| Variable name | Estimate | Standard error | *p*-value | 95% confidence interval | |
|---|---|---|---|---|---|
| Log of juvenile mean abundance one week after treatment | 0.184 | 0.026 | **<0.001** | 0.134 | 0.234 |
| Log of juvenile mean abundance one week after treatment * week | | | **0.033** | | |
| 3 | 0.057 | 0.026 | **0.031** | 0.005 | 0.109 |
| 4 | -0.068 | 0.046 | 0.136 | -0.157 | 0.021 |
| 5 | -0.039 | 0.053 | 0.459 | -0.143 | 0.065 |
| 6 | -0.023 | 0.079 | 0.768 | -0.178 | 0.131 |
| 7 | 0.021 | 0.097 | 0.832 | -0.169 | 0.210 |
| 8 | -0.058 | 0.085 | 0.494 | -0.225 | 0.108 |
| Region (Los Lagos as reference) | 0.169 | 0.047 | **<0.001** | 0.077 | 0.261 |
| Drug used in the on-farm treatment (azamethiphos as reference) | 0.212 | 0.088 | **0.016** | 0.040 | 0.385 |
| Water temperature | 0.047 | 0.014 | **<0.001** | 0.019 | 0.075 |
| Stocking density | 0.017 | 0.006 | **0.002** | 0.007 | 0.028 |
| **Random effects parameters** | | | | | |
| Farm | 0.005 | 0.010 | 0.302 | <0.001 | 0.276 |
| Treatment event | --- | --- | --- | --- | --- |

**Residual structure** (unstructured, non constant variances)

| week | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 2 | 0.354 (0.021) | | | | | | |
| 3 | 0.220 (0.020) | 0.440 (0.028) | | | | | |
| 4 | 0.152 (0.026) | 0.298 (0.033) | 0.741 (0.055) | | | | |
| 5 | 0.112 (0.028) | 0.250 (0.035) | 0.389 (0.047) | 0.701 (0.062) | | | |
| 6 | 0.047 (0.040) | 0.162 (0.052) | 0.276 (0.068) | 0.427 (0.074) | 1.052 (0.119) | | |
| 7 | 0.118 (0.049) | 0.247 (0.064) | 0.395 (0.090) | 0.442 (0.092) | 0.628 (0.120) | 1.379 (0.177) | |
| 8 | 0.100 (0.044) | 0.193 (0.056) | 0.379 (0.082) | 0.346 (0.082) | 0.366 (0.100) | 0.866 (0.134) | 0.936 (0.132) |

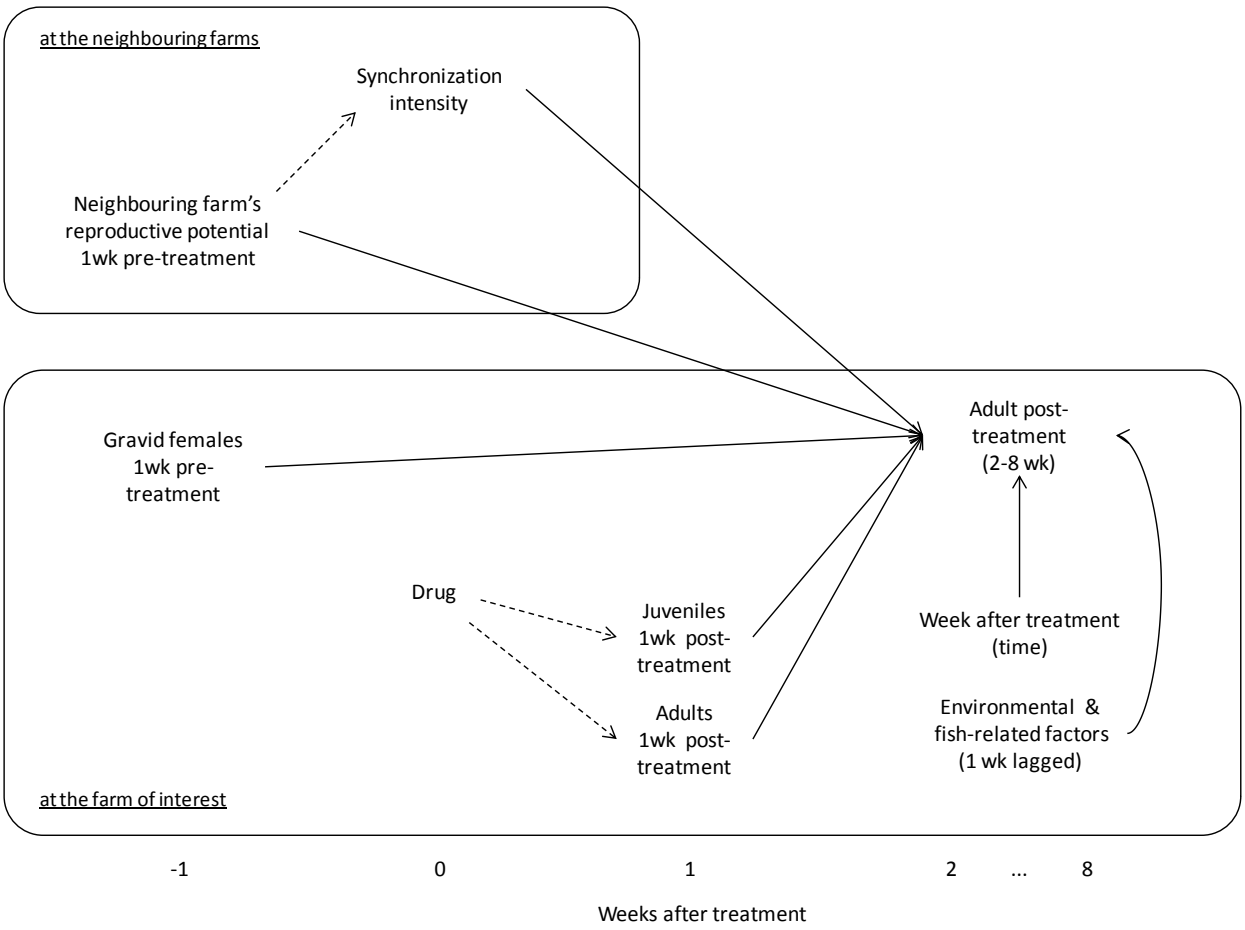


Figure 4.1. Causal diagram for the effect of treatment synchronization on the adult lice abundance between 2 to 8 weeks after treatment. Solid arrows: likely association. Dashed arrows: potential association.

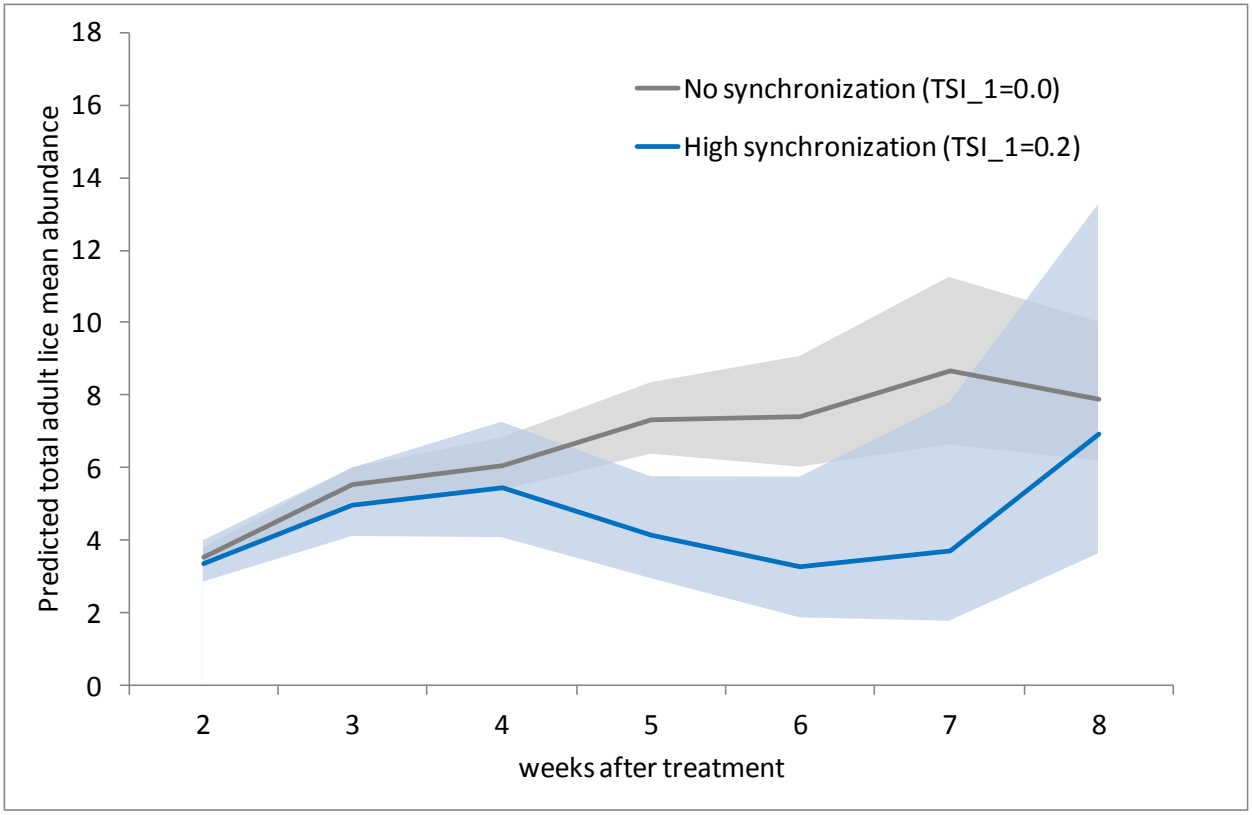


Figure 4.2. Predicted adult lice mean abundance and its 95% CI by week after treatment under no (TSI_1=0.0) and high treatment synchronization (TSI_1=0.2) among neighbouring farms in a 10 km and 1 week synchronizing window. Predictions are estimated from a linear mixed effects model presented in Table 4.3.

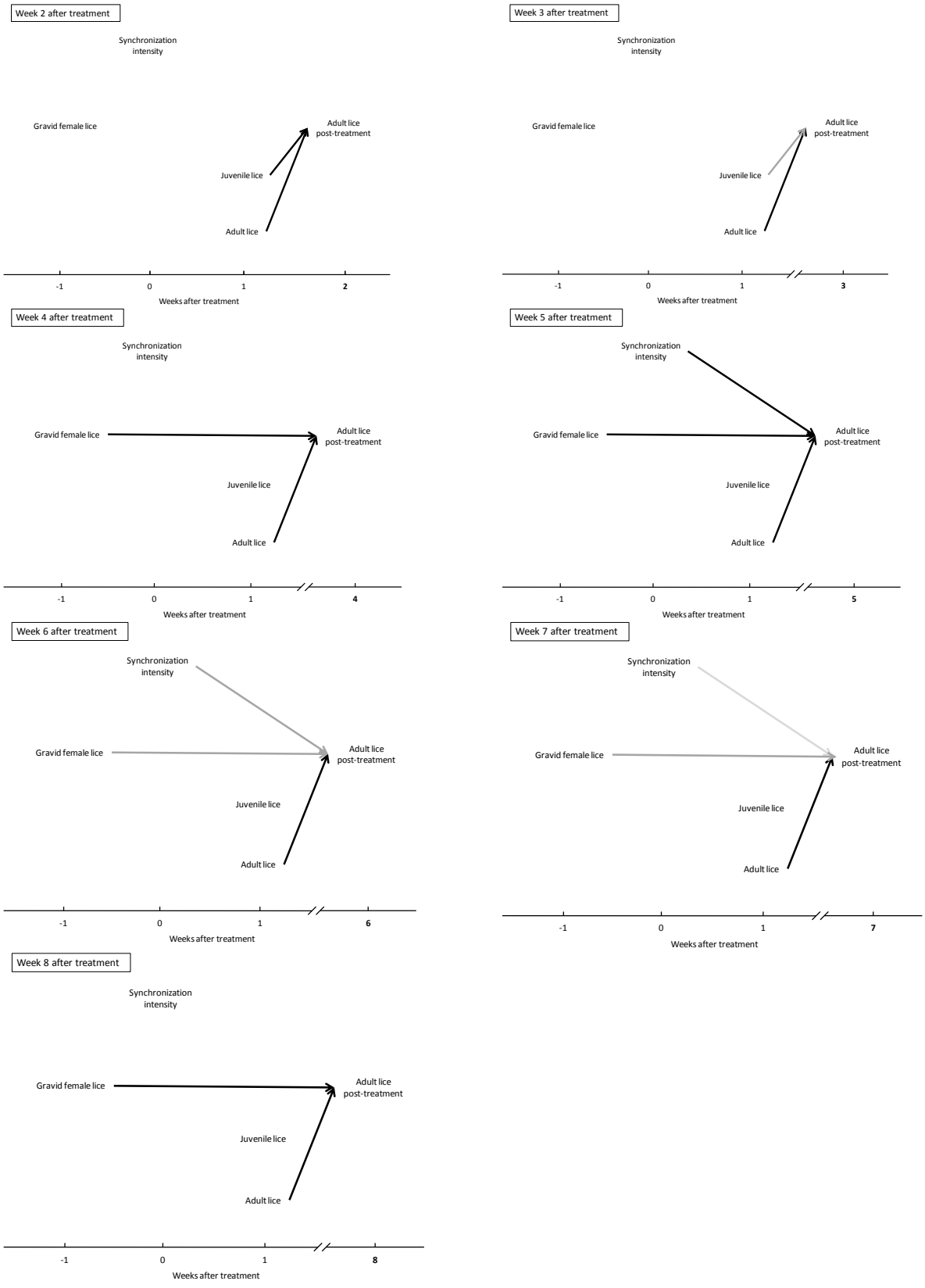


Figure 4.3. Path diagrams for weeks 2 to 8 after treatment synchronization based on the final model for predictors that interacted with time. Remarks: → $p \leq 0.01$; → $0.01 \leq p < 0.05$; → $0.05 \leq p < 0.10$.

## 4.9. Appendix

Table 4.4. Number of correlation parameters, log-likelihood, and the Akaike Information Criteria (AIC) for models with different correlation structures for residuals. AIC difference (ΔAIC) calculated between the current and the top ranked model (unstructured, non-constant variances).

| Correlation structure for residuals | variance and covariance parameters | LL | AIC | ΔAIC (from the top ranked model) |
|---|---|---|---|---|
| Unstructured, non-constant variances | 29 | -2253.5 | 4646.9 | - |
| Autoregressive 1st order, constant variances | 4 | -2388.7 | 4865.5 | 218.6 |
| Toeplitz, constant variances | 9 | -2384.3 | 4866.5 | 219.6 |
| Compound symmetry | 3 | -2475.0 | 5038.0 | 391.1 |

Table 4.5. Descriptive statistics of continuous production and environmental variables included in the model building process.

| Variables | mean | median | Std. dev. | 90% range |
|---|---|---|---|---|
| Gravid female lice mean abundance one week before treatment | 4.13 | 3.00 | 5.60 | 0.80 – 9.75 |
| Adult lice mean abundance one week after treatment | 4.24 | 2.25 | 11.21 | 0.18 – 8.90 |
| Juvenile lice mean abundance one week after treatment | 3.92 | 1.66 | 10.57 | 0.13 – 12.15 |
| Number of neighbouring farms within a 10 km seaway distance (excluding farms with Coho salmon) | 2.61 | 2.00 | 1.51 | 1.00 – 6.00 |
| Number of neighbouring farms that reported immersion treatment within 10 km seaway distance the same week as in the treatment farm | 1.03 | 1.00 | 1.13 | 0.00 – 3.00 |
| Duration (days) of the treatment procedure in the farm (limited to 7 days) | 3.00 | 3.00 | 1.36 | 1.00 – 5.00 |
| Water temperature (°C) | 10.90 | 10.80 | 1.44 | 8.80 – 13.20 |
| Water salinity (ppt) | 30.92 | 31.00 | 2.19 | 27.00 – 33.00 |
| Stocking density ($kg/m^3$) | 7.17 | 6.80 | 3.82 | 1.64 – 13.60 |

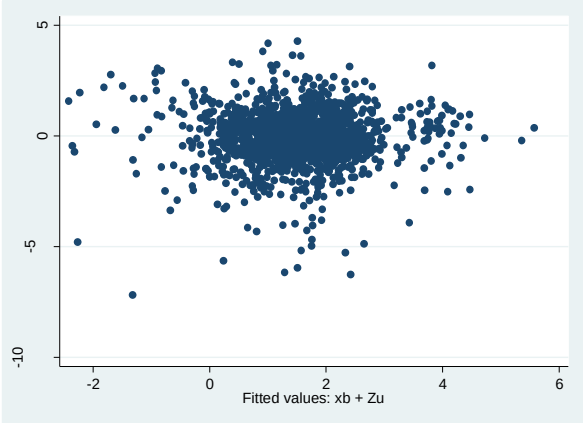


Figure 4.4a. Residual plot for week-level standardized residuals.

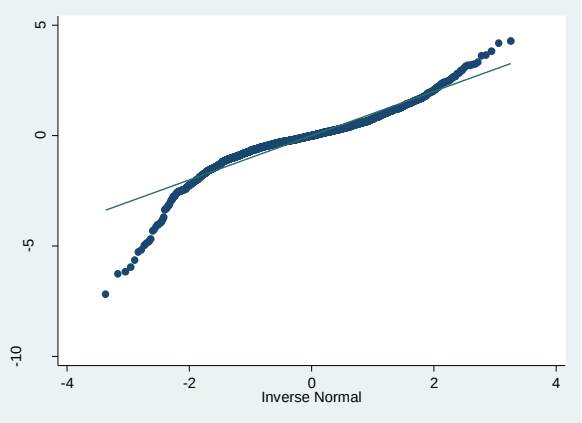


Figure 4.4b. Q-Q plot for week-level standardized residuals.

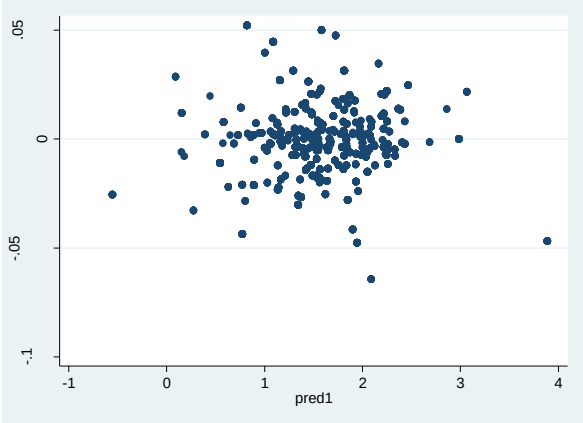


Figure 4.5a. Residual plot for farm-level standardized residuals.

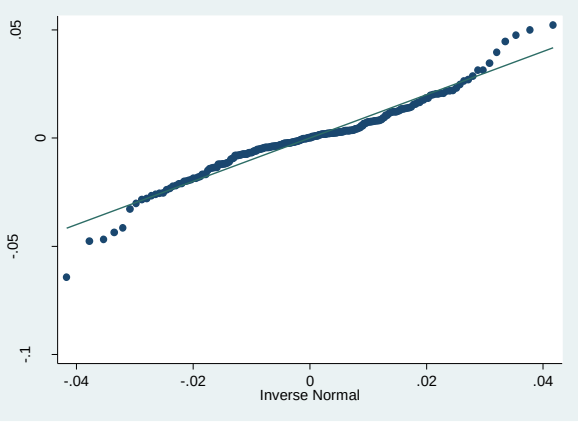


Figure 4.5b. Q-Q plot for farm-level standardized residuals.

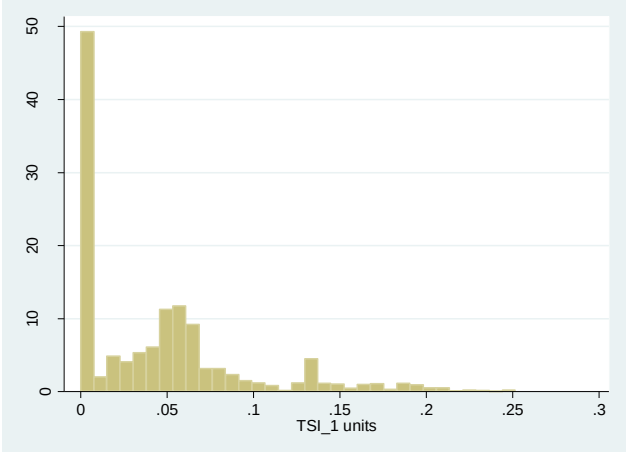


Figure 4.6a. Histogram for TSI_1 values.

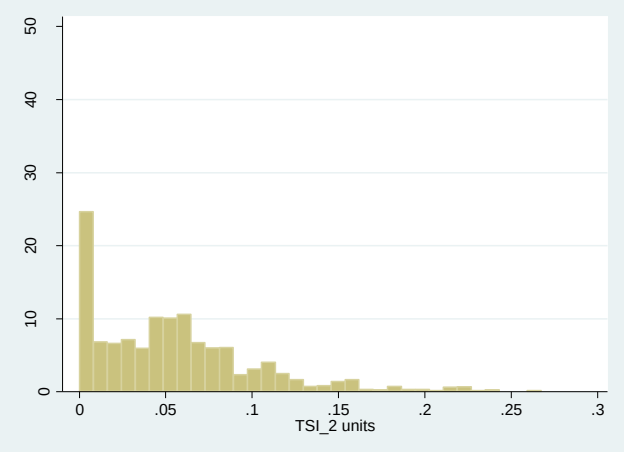


Figure 4.6a. Histogram for TSI_2 values.

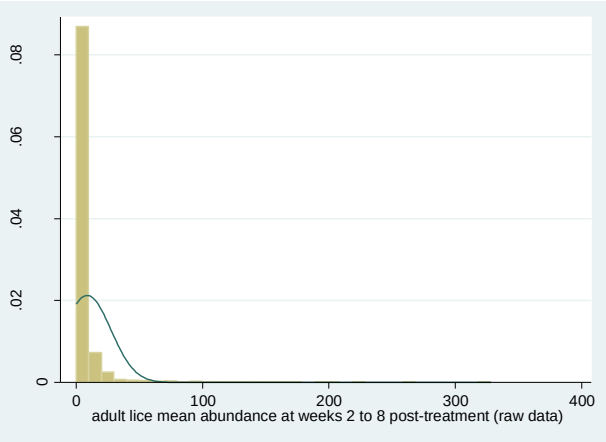


Figure 4.7a. Histogram for adult lice mean abundance at weeks 2 to 8 post-treatment (raw data).

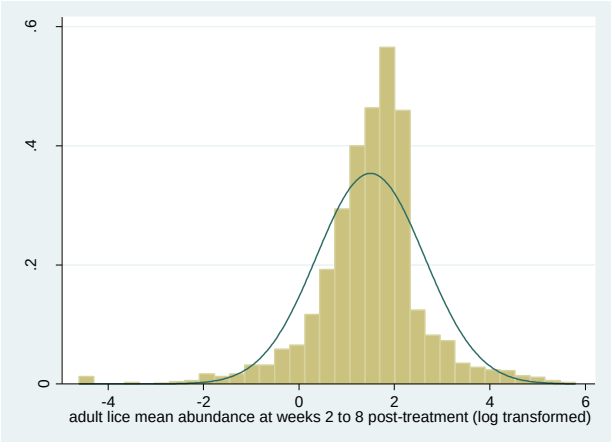


Figure 4.7b. Histogram for adult lice mean abundance at weeks 2 to 8 post-treatment (log transformed).

# CHAPTER 5
# GENERAL DISCUSSION AND CONCLUSIONS

## 5.1. Main findings of the current research

The aims of this thesis were to study the performance of pyrethroid-based treatments on the sea louse *Caligus rogercresseyi* in Chile during 2011 to 2013, and to evaluate the synchronization of treatments as a strategy for improving results at the farm level.

Chapter 1 examined relevant literature regarding pharmacological methods for the control of sea lice associated with farmed salmon and, in particular, methods researchers have used to evaluate the performance of delousing treatments using field data. We also reviewed the main causes of treatment failures, including re-infestation and resistance of sea lice to chemotherapeuntants, and area-level strategies for improving the treatment outcomes on farms. We found that treatment effectiveness can be expressed in different ways, but always involves sea lice levels before and after the treatment procedure. There have been numerous anecdotal reports of treatment failures associated with the use of pyrethroids in Chile; however, information about the causes and geographical extent of these failures is scarce. Apart from that, we verified that coordination/synchronization of delousing treatments is a widely used strategy in salmon-farming countries, even though no formal studies have evaluated its potential benefits, either at the area or the farm level.

In Chapter 2, we evaluated the performance of the three pyrethroid-based products, available in Chile in 2011 and 2012, on different life stages of *C. rogercresseyi*, including juveniles, mobile adults, and gravid female lice. In this study, we modeled post-treatment sea lice level as a function of the drug product and other explanatory variables, using multivariable statistical models. We found that pyrethroids had an intermediate efficacy on adult lice (mean=63%), and a poor performance on juvenile lice (mean=20%). In addition, results showed significant differences in performance among two products in the gravid female group. In the rest of the sea lice stages, the three products were equally effective. We also learnt that environmental and management factors impacted the treatment performance.

In the third chapter, we explored the variation of *C. rogercresseyi* responses to pyrethroid treatments in space and time and examined factors related to this variability. To do this, we modeled the adult lice levels one week after treatment with a linear mixed-effects regression, while controlling for several management and environmental variables, including pre-treatment sea lice levels. Then, we performed a spatial cluster analysis on the farm level predictions, and a spatio-temporal analysis with the treatment level residuals from the multivariable model. Treatment performances were clustered in space; however, clustering did not seem to concentrate at any particular time in the study period. After controlling for predictors two areas of high post-treatment sea lice levels were detected, one in the Los Lagos region and the other in the Aysén region. In this chapter we learnt that the performance of pyrethroids observed in the study period was driven by unknown factors that clustered in space. Factors that may be responsible for these patterns are low sensitivity of sea lice to pyrethroids, different treatment modalities and drug administration issues; however, further research should be done first to confirm these possibilities.

In Chapter 4, we used a repeated measures linear mixed-effects model to evaluate the effect of synchronized treatments, within one week and 10 km seaway distance, on sea lice abundance at the farm level from week 2 to 8 after treatment. Results indicated that treatment synchronization was significantly associated with lower adult lice levels at weeks 5 and 6 post-treatment. These findings suggest that synchronization can improve immersion treatment performance by keeping sea lice levels low for longer periods of time.

## 5.2. Methodological aspects

One common methodological aspect across the studies in this thesis was the measure of treatment performance. In general, treatment success is evaluated by comparing sea lice levels before and after the procedure, which is called efficacy or effectiveness. In practice, treatment efficacy has been expressed as percent reduction ([pre-treatment – post-treatment] / pre-treatment) (Branson et al., 2000; Gustafson et al., 2006; Jimenez et al., 2012). Although this expression is easy to interpret, it can produce undetermined

values when the pre-treatment level is zero, with the consequent loss of data. An expression of efficacy that overcomes the former of these problems is the ratio between the post-treatment level and the pre-treatment sea lice level (post-treatment / pre-treatment) (Jones et al., 2012, 2013). Values greater than one are achieved when the post-treatment level is greater than pre-treatment. Treatment performance has been also evaluated in a multivariable way; in that case, the post-treatment sea lice level is modeled as a function of the pre-treatment sea lice level (Lees et al., 2008a,b).

In our three studies, we calculated treatment performance using multivariable analyses, which permitted us to have a coefficient for the pre-treatment level and measure the magnitude, direction, and significance of the effect. In chapters 2 and 3, we measured post-treatment sea lice levels only once post-procedure, while in Chapter 4 we recorded up to 7 weekly, post-treatment measurements. In all cases, we recorded pre-treatment levels at a single moment before the procedure.

Multivariable techniques in the evaluation of treatment performance are useful because they permitted us to compare treatment outcomes under very different management and environmental conditions. These techniques also allowed us to control for factors not under the control of the farm manager (i.e., environmental) and provided an opportunity to learn about the impacts of factors related to farm practices (e.g. fish stocking density). Linear regression, for example, has been applied to control for extraneous variables when the objective was to evaluate the effect of a particular factor, as in Jones et al. (2012), who wanted to determine whether changes in effectiveness of emamectin benzoate were present from 2004 to 2008 in the Bay of Fundy (NB, Canada). In other cases, multivariable techniques are used out of more exploratory interest, for example, to examine factors associated with the efficacy of treatment interventions (Lees et al., 2008a).

In our case, the multivariable approach was applied from the study design to the data analysis. For each study, we built a causal diagram to determine which factors we should include in the data analysis in

order to control for potential confounding factors. Because *C. rogercresseyi* is a parasite that has a life cycle with 8 developmental stages (González & Carvajal, 2003), when we modeled the sea lice level at a particular week (*t*), we usually tested the effect of the abundance of the current and previous life stages on the farm for the previous week (*t*–1). For example, in Chapter 4 we were interested in factors that impact post-treatment adult lice levels. We know that juvenile and adult lice can survive the treatment, and that juvenile lice (chalimus III and IV) at time *t*–1 may evolve into adults at time *t*, so we incorporated both adult and juvenile lice levels at one week before treatment.

In most cases, our decision to include pre-treatment levels at the farm of interest and/or at neighbouring farms was informed by the *C. rogercresseyi* life cycle dynamics (González and Carvajal, 2003). For our estimations of lice development in time we considered water temperature as 11 °C, which was the mean value in our dataset. For example, in studies aiming to model adult lice levels (models for mobile adults in Chapter 2, and models for Chapter 3) we included levels of juveniles and adults one week before treatment. The juvenile group represents individuals that will evolve into adult lice during the time between the pre- and the post-treatment samples (~ 15 days) (Table 1.1). The adult group, in turn, mainly embodies adult lice that survived the treatment and remained as adults at the post-treatment sample. In the special case of Chapter 4, we made an exception as we included juvenile and adult lice levels one week after the procedure. The reason for that is we were interested in modeling the raise of sea lice levels within an 8-week period after treatment; consequently we presumed the 1-week post-treatment levels would have substantial impact on sea lice levels from week 2 to 8 post-treatment, and, in a sense, represented the output of the treatment. In addition, because adult lice levels were followed for a relatively long period of time (i.e. 8 weeks post-treatment), we had enough time for evaluating the potential impact of on-farm gravid females one week before treatment due to their offspring which could eventually evolve into adult lice in approximately 35 days (Table 1.1).

We used different measures for the external infectious pressure which varied in complexity. For example, in Chapter 2 we measured it as the number of neighbouring farms within 30 km seaway distance, which is a fairly simple way to measure this component. In this case we did not consider the time distance a larva needs to travel from neighbouring farms, settle and evolve into adult lice, but we just assumed neighbouring farms as a "constant" source of lice over time.

In Chapter 3, we increased the complexity of this measure by a distance weighted sum of the gravid female abundance at neighbouring farms within 30 km seaway distance; however, due to practical issues we estimated this external component at two weeks before treatment, which is not the ideal time frame for evaluating the evolution of larvae into adult lice (Table 1.1). Despite this drawback, the external infectious pressure measure (i.e. NRP) was significantly associated with our outcome, which may suggest that a NRP value at a certain week is explained by NRP at a consecutive week at some extent (i.e. consecutive NRP measures are correlated in time).

In Chapter 4 we calculated the external infectious pressure the same way as in Chapter 3, but we measured it at one week before treatment. The extended time period we followed the adult lice levels (i.e. 2 to 8 weeks) provided us the necessary time (Table 1.1) to evaluate the evolution of larvae originated in neighbouring farms into mature adult lice at the farm of interest.

It is important to recall that in Chapter 4 we were interested in evaluating the treatment synchronization effect over a 7-week time period after treatment. Based on the sea lice life cycle and the potential effect of treatments at the neighbouring farms, we expected the effect of synchronization would not be constant over time, but that it would be evident at certain weeks after treatment. We expected a similar time-dependent effect with other sea lice predictors included in the model, such as pre-treatment sea lice levels at the farm of interest and at neighbouring farms. We managed these time-dependent effects by including interactions with time (i.e. weeks post-treatment) and, with this analytical procedure, we were able to

determine the particular weeks at which the effect was significant.

We would like to emphasize that, although treatment synchronization is a common practice in most salmon-farming regions in the world, there is only one published study that has attempted to evaluate coordinated treatments (Wadsworth, 1998). Consequently, we had to figure out a reliable way to measure treatment synchronization. To this end, we tested several measures of synchronization, and found the measure that best fitted our data was the total number of farms that treated within a spatio-temporal window after we estimated the total number of gravid females in the area. Because we were aware that the effect of distance between farms was important (Jansen et al., 2012; Kristoffersen et al., 2013), we weighted the number of treated neighbouring farms by seaway distance from the farm of interest. This improved substantially the model fit.

The first published study of spatial autocorrelation among residuals from a model dealing with the response of sea lice to chemotherapeutants was Wescott et al. (2008). The rationale behind their analysis was to remove known sources of variability from the observed variation in the sensitivity of *L. salmonis* to emamectin benzoate in order to detect second order spatial effects (Pfeiffer et al., 2008), which may suggest transmission of resistance genes between fish farms. We used a similar methodology to evaluate the geographic distribution of pyrethroid-based responses. Because in most cases we included more than one treatment per farm in the analysis, we evaluated the clustering at both the farm and at the treatment level, by subjecting farm effect predictions and residuals to a purely spatial and to a spatio-temporal analysis, respectively. To our knowledge this is the first time that this methodological approach has been applied to the evaluation of field performance of delousing treatments.

### 5.3. Other findings

Recent research has shown that external infectious pressure is a key factor that impacts sea lice abundance at the farm level (Jansen et al., 2012; Kristoffersen et al., 2013). We have accounted for this in each of the

three studies in two different ways. In Chapter 2, we used a relatively simple measure, which was the total number of farms rearing susceptible salmonid species (Atlantic salmon and rainbow trout) within a 30 km seaway distance. For chapters 3 and 4, in contrast, we summed the gravid female mean abundance at each neighbouring farm within a 30 km seaway distance of the farm of interest. As per Jansen et al. (2012) and Kristoffersen et al. (2013), we also weighted each neighbouring farm's contribution by the seaway distance from the farm of interest.

It is important to note that our measures of external infectious pressure were highly significant, which confirms the importance of this factor in the sea lice abundance at a farm. It also shows how this factor significantly impacts the treatment performance and, even, treatment synchronization; therefore, it should always be considered in treatment performance assessment. Our choice for using a Gaussian kernel density function to represent the transmission of sea lice between farms was influenced by a study by Kristoffersen et al. (2013) who found that this distribution described appropriately the transmission of sea lice in Chile. However, a study carried out in Norway has found the exponential function is much more appropriate to model the local transmission of sea lice (Aldrin et al., 2013). As we only tried the Gaussian kernel function we do not know whether other functions may better represent sea lice transmission in Chile. Further research should be done to address this issue.

In Chapter 2 we evaluated treatment performance at the cage level, which permitted us to evaluate with greater precision the effect of timing of the post-treatment sea lice measure on the treatment outcome. Although it was not the first time this factor has been considered in treatment performance evaluation (see Lees et al., 2008a), its inclusion as a continuous variable in Chapter 2, gave a clearer insight into its role in the quantification of treatment performance. A similar treatment to this variable was given by Jimenez et al. (2013) in a study aimed to evaluate treatment efficacy on *L. salmonis* with a multivariable approach.

It has been suggested that the longer it takes to treat a whole farm, the greater the post-treatment sea lice abundance will be (Costello, 2006). Consequently, we tested this variable in chapters 3 and 4 in order to control for its potential effect. We found treatment duration was significant (Chapter 3) which provides the first evidence of its effect. The non-significant effect in Chapter 4 may be due to the fact that the first week after treatment was not considered in the outcome, in this study, but was included as one of the predictors.

## 5.4. Impacts

This research provides new evidence-based knowledge to the Chilean salmon industry regarding the efficacy of pyrethroids for the control of sea lice. We found that by 2011-2012, on average, pyrethroids were effective to some extent on adult stages of *C. rogercresseyi*; and that juvenile stages seemed to be less sensitive to these drugs. This finding suggests that pyrethroids should not be used as the only drug for controlling sea lice. This is the first study that has addressed this issue using field data, and controlling for factors that may confound the evaluation of treatment performance.

It is important to note that SAG, the Chilean authority responsible for the regulation of animal drugs, has authorized cypermethrin-based products for its use against both juvenile and adult *C. rogercresseyi*, while deltamethrin-based products are approved only for adult stages (SAG, 2013). Our results suggest both drugs have a similar effect on juvenile stages. This finding supports a re-evaluation of the efficacy of pyrethroid-based products on juvenile *C. rogercresseyi*, by the Chilean authority.

This research also identified two areas where pyrethroids had poorer performance and we have discussed potential causes for this effect. The methodology used in this study did not allow us to determine the causes of the clustering. Potential factors that may explain this situation are increased tolerance to pyrethroids and problems with the administration of the drug, among others; however, further research is needed to confirm or rule out these possibilities. The methodological approach used in Chapter 3 shows

how regular surveillance information, involving sea lice levels, management, and environmental factors, may be used to identify risk areas, so that other analytical tools (i.e. bioassays) may be targeted to confirm or rule out developing of resistance.

In addition, this research provided initial, strong evidence for the positive effect of treatment synchronization on sea lice abundance at the farm level. We have attempted to explain the biological mechanisms involved in this procedure, and have discussed the procedure's potential and limitations. Because we found that treatment synchronization appeared to delay the rise of sea lice levels after a treatment procedure, the most evident potential impact of this practice would be a delay of the need for the next treatment by 2 or 3 weeks, which may reduce the total number of treatments per production cycle. This would have a major impact on farm costs and the marine environment. Our findings also suggest that one bath treatment even if done in synchrony is not adequate to eliminate sea lice. We hypothesize that this is due to the complicated life cycle of sea lice and the inability of bath treatments to target all stages.

### 5.5. Other data issues

As a general comment, it is worth to mention that, like in any monitoring system, the sampling scheme is a key element for generating representative information and for valid inference. In general, sampling schemes in sea lice monitoring combine statistical (i.e. variance of sea lice within and between pens) and practical (i.e. labour and costs) criteria, and they are always subjected to a degree of sampling error. Another source of potential bias is related to the fact that in aquaculture sampling rarely is truly random; this is because fish are not individually tagged and the fish catch itself may be a complex procedure. This particular situation may also increase the sampling error. Regarding these sampling issues, we do not have any information that allows us to quantify the sampling error of the data we used in this thesis.

In chapter 3, sea lice levels showed an unusual pattern over time characterized by a remarkable concentration of data points below approximately 8 adult lice/fish (see Figures 3.7a/b and 3.14b). This pattern may be a consequence of the treatment trigger threshold set in 6 adult lice per fish by the Chilean legislation, but it may also denote some sort of underestimation as farms with more than 9 adult lice per fish for more than 3 consecutive weeks may be exposed to radical control measures such as anticipated harvest. This situation may have biased our results to some extent.

## 5.6. Conclusions and future directions

Between 2011 and 2013, pyrethroids were widely used in Chile to combat sea lice. Our results suggest that, on average, pyrethroids showed intermediate to low efficacy against adult sea lice. In general, pyrethroid-based products showed similar performance in mobile stages, with the exception of the gravid female lice that seemed more sensitive to cypermethrin. We found that pyrethroid performance was significantly impacted by environmental and management factors, but also by unknown factors, possibly related to sea lice sensitivity to pyrethroids, treatment modalities or aspects of drug administration; however, further research needs to be carried out to elucidate this situation. Finally, we provided strong evidence for the positive effect of treatment synchronization on reducing the sea lice abundance at the farm level.

Results of this thesis have provided new knowledge which can be used to optimize the use of synthetic pyrethroids for controlling *C. rogercresseyi* in Chile. In Chapter 2 we have found that the main target for pyrethroids are adult stages of *C. rogercresseyi*, the juvenile lice being less sensitive to this drug. Consequently, farmers should no longer consider pyrethroids as a unique option for managing sea lice. In Chapter 3 we found there are unknown factors (i.e. different from factors that the Chilean salmon industry regularly records) driving a spatial clustering of treatment performance. This is a warning that the Chilean salmon industry must consider carefully, because potential drivers of that spatial clustering might be related to development of resistance in sea lice to pyrethroids. This result highlights the need for a

sensitivity/resistance monitoring system. Finally, in Chapter 4 we provided strong evidence that the effect of pyrethroids in a farm may be substantially improved if treatments are synchronized in the area. This area-level procedure may reduce the number of treatments in the production cycle. This evidence should be considered by the salmon industry in order to strongly encourage treatment synchronization among farms.

The role of epidemiology, as an observational discipline, is to guide complementary research by narrowing the potential drivers of the phenomena under study. In this work, we have used regular surveillance data to address treatment performance, and provided new knowledge about the magnitude of the problem, its geographic extent, and potential causes. Further research should be oriented towards confirmation of resistance development to pyrethroids and, if possible, to delimit the more affected areas. In addition, the efficacy of other drugs currently used in Chile, such as azamethiphos, should also be evaluated.

Keeping in mind that synchronization of treatments within a one-week and a 10 km-window appeared to have a positive impact on sea lice levels, the next step would be to evaluate whether better results can be achieved by modifying the synchronization window. Ultimately other strategies that target juvenile stages and new infections in combination with bath treatments should be evaluated as they may be more effective given the rapid increase in sea lice observed shortly after treatment especially in areas where treatments were not synchronized.

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